

-1-

Date: <u>7-24-03</u> Express Mail Label No. <u>EV 05203/082 US</u>
--

Inventors: H. William Harris, Jr., David R. Russell,
Jacqueline Nearing, and Marlies Betka
Attorney's Docket No.: 2213.1004-012

METHODS FOR RAISING PRE-ADULT ANADROMOUS FISH

RELATED APPLICATIONS

This application is a divisional of U.S. Application No. 10/270,876, filed October 11, 2002, which is a divisional of U.S. Application No. 09/687,477, filed 5 October 12, 2000, now U.S. Patent No. 6,463,883. The entire teachings of the aforementioned applications are incorporated herein by reference.

BACKGROUND OF THE INVENTION

In nature, many anadromous fish live most of their adulthood in seawater, but swim upstream to freshwater for the purpose of breeding. As a result, anadromous fish 10 hatch from their eggs and are born in freshwater. As these fish grow, they swim downstream and gradually adapt to the seawater.

Fish hatcheries have experienced difficulty in raising these types of fish because the window of time in which the pre-adult fish adapts to seawater (e.g., undergoes smoltification) is short-lived, and can be difficult to pinpoint. As a result, these 15 hatcheries experience significant morbidity and mortality when transferring anadromous fish from freshwater to seawater. Additionally, many of the fish that do survive the transfer from freshwater to seawater are stressed, and consequently, experience decreased feeding, and increased susceptibility to disease. Therefore, these anadromous fish often do not grow well after they are transferred to seawater.

The aquaculture industry loses millions of dollars each year due to problems it encounters in transferring pre-adult anadromous fish from freshwater to seawater. Hence, a need exists to improve methods involved in transferring pre-adult anadromous fish to seawater. A further need exists to increase survival and growth, and reduce stress, of pre-adult anadromous fish that have been transferred to seawater.

SUMMARY OF THE INVENTION

The present invention relates to methods for improving the raising of pre-adult anadromous fish or preparing these fish for transfer to seawater by increasing expression of a receptor, referred to as the Polyvalent Cation Sensing Receptor (PVCR). The expression and/or sensitivity of the PVCR is increased by subjecting the pre-adult anadromous fish to at least one modulator of the PVCR. The pre-adult anadromous fish are subjected to the modulator when it is added to their freshwater environment, and optionally, to the feed. The invention encompasses adding at least one PVCR modulator to the freshwater, and adding feed for fish consumption to the freshwater. The feed contains sodium chloride (NaCl) and, optionally, at least one PVCR modulator in an amount to contribute to a significantly increased level of the PVCR modulator in the serum of the pre-adult anadromous fish. Increased expression and/or sensitivity of the PVCR is maintained until the fish are ready to be transferred to seawater. The pre-adult anadromous fish can be maintained in the freshwater having at least one PVCR agonist until they are ready to be transferred to seawater.

In one embodiment of the invention, pre-adult anadromous fish (e.g., salmon, trout and arctic char) are prepared for transfer from freshwater to seawater by adding PVCR agonists, such as calcium and magnesium to the freshwater, and adding feed for fish consumption having between about 1% and about 10% NaCl by weight (e.g. between about 10,000 mg/kg and 100,000 mg/kg) to the freshwater. The amount of calcium added to the freshwater is an amount sufficient to bring the concentration up to between about 2.0 mM and about 10.0 mM, and the amount of magnesium added is an amount sufficient to bring the concentration up to between about 0.5 mM and about

10.0 mM. The feed can optionally include a PVCR agonist, such as an amino acid. A particular amino acid that can be added is tryptophan in an amount between about 1 gm/kg and about 10 gm/kg. The present invention also includes, optionally, exposing the pre-adult anadromous fish to a photoperiod. Preferably, the photoperiod is
5 continuous (e.g., for a continuous period of between about 12 hours and about 24 hours in a 24 hour period).

Additional embodiments of the invention include methods of increasing or improving food consumption before and/or after seawater transfer, increasing growth, increasing survival and/or reducing mortality, reducing osmotic damage, transferring
10 parr (e.g., between about 10 and about 60 grams) to seawater, and transferring a pre-adult anadromous fish to seawater having a temperature of about 14°C to about 19°C. These methods are performed by adding at least one PVCR modulator to the freshwater, subjecting or exposing the pre-adult anadromous fish to at least one PVCR modulator, or introducing the pre-adult anadromous fish to freshwater having at least one PVCR
15 modulator, in an amount sufficient to increase expression and/or sensitivity of the PVCR. The methods also involve adding feed having between about 1% and about 10% NaCl by weight to the freshwater and transferring the pre-adult anadromous fish to seawater.

In other embodiments, the invention encompasses detection assays or methods
20 of determining whether a pre-adult anadromous fish, that are subjected to at least one PVCR modulator and are fed with feed having between about 1% and about 10% NaCl by weight, are ready for transfer to seawater, by assessing the amount of PVCR expression in the pre-adult anadromous fish. An increased level of expression and/or sensitivity, as compared to a control (e.g., PVCR expression from a fish not subjected to
25 a PVCR modulator), indicates that the pre-adult anadromous fish are ready for transfer to seawater. In a preferred embodiment, the assay includes contacting an anti-PVCR antibody with a sample (e.g., gill, skin, intestine, urinary bladder, kidney, brain or muscle) under conditions sufficient for the formation of a complex between the antibody and the PVCR; and detecting the formation of the complex. In another

embodiment the assay relates to hybridizing a nucleic acid sequence having a detectable label to the nucleic acid sequence of the PVCR of a sample taken from the pre-adult anadromous fish and detecting the hybridization.

In yet another embodiment, the present invention relates to various compositions and mixtures. In particular, the invention pertains to an aquatic food composition having a concentration of NaCl between about 10,000 mg/kg and 100,000 mg/kg (e.g., about 12,000 mg/kg). The aquatic food composition can optionally include a PVCR modulator (e.g. tryptophan in an amount between 1 gm/kg and 10 gm/kg).

The invention also embodies an aquatic mixture for providing an environment to improve the raising of pre-adult anadromous fish. The mixture includes at least one PVCR modulator. An example of such a mixture is a calcium source, that when added to freshwater, provides a concentration of between about 2.0 mM and about 10.0 mM; and a magnesium source, that when added to freshwater, provides a concentration of between about 0.5 mM and 10.0 mM.

In yet another embodiment, the present invention relates to kits. In particular, the invention embodies kits for improving the raising of pre-adult anadromous fish, that includes a PVCR modulator for addition to the freshwater and an aquatic food composition, as described herein. In another embodiment, the invention includes kits for determining whether a pre-adult anadromous fish are ready for transfer to seawater, after being subjected to at least one PVCR modulator and feed having between about 1% and about 10% NaCl by weight. The kit includes either an anti-PVCR antibody, and a solid support; or a nucleic acid sequence having a detectable label that can hybridize to nucleic acid of an aquatic PVCR.

Surprisingly, it has been discovered that increased expression and/or altering the sensitivity of the PVCR allows these pre-adult anadromous fish to better adapt to seawater. Until the discovery of the present invention, the aquaculture industry was unable to transfer the pre-adult anadromous fish to seawater without subjecting the fish to stress, death and/or disease. Unlike this practice, carrying out the steps of the invention increases the expression and/or alters the sensitivity of the PVCR and allows

for transfer of the pre-adult anadromous fish to seawater with minimal or no stress, death and/or disease, and unexpectedly provides several benefits, such as increased growth and the ability to transfer these fish to water having higher temperatures, as further described herein. The present invention results in one or more of the following

5 advantages in transferring pre-adult anadromous fish to seawater: a reduction in mortality; improvement in feeding; an increase in growth; a decrease in the amount of diseased fish; and/or a reduction in osmotic shock. The present invention also allows for earlier harvesting of the fish with increased flexibility in producing fish year round. Additionally, the methods of the present invention can result in significant cost savings

10 for fish hatcheries.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram illustrating the partial nucleotide (SEQ ID NO:1) and amino acid (SEQ ID NO:2) sequences of the polyvalent cation-sensing receptor (PVCR) of Atlantic salmon (*Salmo salar*).

15 Figure 2 is a diagram illustrating the partial nucleotide (SEQ ID NO:3) and amino acid (SEQ ID NO:4) sequences of the PVCR of arctic char (*Salvelinus alpinus*).

Figure 3 is a diagram illustrating the partial nucleotide (SEQ ID NO:5) and amino acid (SEQ ID NO:6) sequences of the PVCR of rainbow trout (*Oncorhynchus mykiss*).

20 Figures 4A-B are diagrams illustrating the alignment of the amino acids sequences for shark kidney cation receptor ("SKCaR") (SEQ ID NO: 18), salmon (SEQ ID NO:2), arctic char (SEQ ID NO:4) and rainbow trout (SEQ ID NO:6).

Figure 5 is a schematic diagram illustrating industry practice for salmon aquaculture production, prior to the discovery of the present invention. The diagram

25 depicts key steps in salmon production for S0 (75 gram) and S1 (100 gram) smolts. The wavy symbol indicates freshwater while the bubbles indicate seawater.

Figure 6 is a graphical representation illustrating length (cm) and weight (gm) of APS Process I Smolts 50 days after ocean netpen placement. APS Process I smolts had

an average weight of 76.6 gram when placed seawater and were sampled after 50 days. APS Process I is defined is Example 2.

Figure 7 is a graphical representation illustrating length (cm) and weight (gm) of representative APS Process I smolts prior to transfer to seawater.

5 Figure 8 is a graphical representation illustrating length (cm) and weight (gm) of APS Process I smolts before transfer, and mortalities.

Figure 9 is a three dimensional graph illustrating the survival over 5 days of Arctic Char in seawater after being maintained in freshwater, APS Process I for 14 days, and APS Process I for 30 days.

10 Figure 10 is a graphical representation illustrating the length (cm) and weight (gm) of St. John/St. John APS Process II smolts prior to seawater transfer. APS Process II is defined in Example 2.

Figures 11A and 11B are graphical representations illustrating weight (gm) and length (cm) of APS Process II smolt survivors and mortalities 5 days after transfer to
15 seawater tanks, and 96 hours after transfer to ocean netpens.

Figure 12 is a graphical representation showing weight (gm) and length (cm) of APS Process II smolt mortalities after 5 days after transfer to ocean netpens.

Figures 13A-G are photographs of immunocytochemistry of epithelia of the proximal intestine of Atlantic Salmon illustrating PVCR localization and expression.

20 Figure 14 is a photograph of a Western Blot of intestinal tissue from salmon maintained subjected to APS Process I for immune (lane marked CaR, e.g., a PVCR) and preimmune (land marked preimmune) illustrating PVCR expression.

Figure 15 is a photograph of a Western Blot of intestinal tissue from trout fingerlings for immune (lane marked CaR, e.g., a PVCR) and preimmune.(lane marked
25 preimmune) illustrating PVCR expression.

Figures 16A-H are photographs of immunocytochemistry of epithelia of proximal intestine of rainbow trout using anti-PVCR antiserum illustrating PVCR localization and expression.

Figure 17 is a photograph of a Western Blot comparing levels of PVCR of fish in freshwater, water having calcium and magnesium, and seawater, illustrating PVCR expression.

Figures 18A-C are photographs of immunolocalization of the PVCR in the epidermis of salmon illustrating PVCR localization and expression.

Figure 19 is a schematic drawing illustrating adaptive changes of fish in seawater and in freshwater.

Figure 20 is a graphical representation of serum calcium concentrations (mM) over time in rainbow trout subjected to transfer to either seawater or water mixture of the present invention. All data points represent a least 5 independent determinations mean \pm standard deviation from a single representative experiment.

Figure 21 is a graphical representation showing increases in serum calcium concentrations (mM) over time induced by feeding trout maintained in a water mixture (3mM calcium, 1mM magnesium) and a standard freshwater pelleted diet containing additional 1% sodium chloride (w/w).

Figures 22A and 22B are graphical representations of alterations in serum calcium (Figure 22A) and sodium (Figure 22B) after seawater transfer of S1 Atlantic salmon smolts.

Figures 23A and B are graphical representations of serum calcium, magnesium and sodium levels (mM) over time from Atlantic Salmon S1 APS Process I treated fish. Each value displays the mean \pm S.D. of a minimum of 10 independent determination from this single representative experiment.

Figure 24 is a graphical representation illustrating the weight (gm) and length (cm) of representative APS Process II smolts prior to transfer to seawater. This representative sample (n=100) of APS Process II smolts possess a wide range of body weights (3.95-23 gram) with an average body weight of 11.5 gm. Note that all mortalities (n=10) occurred only in the smaller fish in the transfer group.

Figure 25 is a graphical representation illustrating the quantitation of serum concentrations (mM) of calcium, magnesium and sodium in preadult Atlantic salmon

subjected to APS Process II after their transfer to seawater. All values shown are the mean+ S.D. of a minimum of 10 independent samples from a single representative experiment.

Figures 26A-C are an alignment illustrating nucleic acid sequences for the
5 PVCR of Atlantic Salmon (SEQ ID NO.: 1), Char (SEQ ID NO.: 3), Chum Salmon (SEQ ID NO.:7), Coho Salmon (SEQ ID NO.:9), King Salmon (SEQ ID NO.:11), Pink Salmon (SEQ ID NO.:13), Sockeye Salmon (SEQ ID NO.:15) and Trout (SEQ ID NO.: 5).

Figures 27A-B are an alignment illustrating the open reading frame of the
10 polypeptide sequences for the PVCR of Atlantic Salmon (SEQ ID NO.: 2), Char (SEQ ID NO.: 4), Chum Salmon (SEQ ID NO.: 8), Coho Salmon (SEQ ID NO.: 10), King Salmon (SEQ ID NO.: 12), Pink Salmon (SEQ ID NO.: 14), Sockeye Salmon (SEQ ID NO.: 16) and Trout (SEQ ID NO.: 6).

Figures 28A-B are a diagram illustrating the nucleic acid sequence of SKCaR
15 (SEQ ID NO.: 17).

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods for improving the raising of pre-adult anadromous fish and/or methods for preparing pre-adult anadromous fish for transfer from freshwater to seawater. The methods involve increasing expression and/or altering
20 the sensitivity of a Polyvalent Cation Sensing Receptor (PVCR) (e.g., at least one PVCR). The invention relates to increasing expression of the PVCR that affects the fish's ability to adapt to seawater (e.g., to smolt), to undergo smoltification, to survive, to increase growth, to increase food consumption, and/or to be less susceptible to disease.

25 In particular, the methods of the present invention include adding at least one PVCR modulator to the freshwater, and adding a specially made or modified feed to the freshwater for consumption by the fish. The feed contains a sufficient amount of sodium chloride (NaCl) (e.g., between about 1% and about 10% by weight, or about

10,000 mg/kg to about 100,000 mg/kg) to significantly increase levels of the PVCR modulator in the serum. This amount of NaCl in the feed causes or induces the pre-adult anadromous fish to drink more freshwater. Since the freshwater contains a PVCR modulator and the fish ingest increased amounts of it, the serum level of the PVCR
5 modulator significantly increases in the fish, and causes increased PVCR expression and/or altered PVCR sensitivity. This process allows the pre-adult anadromous fish to be “pre-conditioned” and better adapt to seawater.

The methods of the present invention pertain to adapting pre-adult anadromous fish to seawater. Anadromous fish are fish that swim from seawater to freshwater to
10 breed. Anadromous fish include, for example, salmon (e.g., Atlantic Salmon (*Salmo salar*), Coho Salmon (*Oncorhynchus kisutch*), Chinook Salmon (*Oncorhynchus tshawytscha*), Chum Salmon (*Oncorhynchus keta*), Pink Salmon (*Oncorhynchus gorbuscha*)), char (e.g., Arctic Char (*Salvelinus alpinus*)) and trout (e.g., Rainbow Trout (*Oncorhynchus mykiss*)). Anadromous fish also include fish that are unable to
15 swim to seawater (e.g., landlocked), but have the physiological mechanisms to adapt to seawater. The term “pre-adult anadromous fish,” as used herein, refers to anadromous fish that have not yet adapted to seawater. These fish are generally juvenile fish. Pre-adult anadromous fish include, but are not limited to fish that are fingerlings, parr or smolts. As used herein, a “smolt” is a fish undergoing physiological changes that allows
20 the fish to adapt to seawater, or survive upon subsequent transfer to seawater. The term, “smolt,” also refers to a fish that is not at the precise developmental stage to survive uninjured upon transfer to seawater, but rather is one of a population of fish wherein, based on a statistical sampling and evaluation, the population of fish is determined to be at a physiological stage ready for transfer to seawater.

25 The present invention includes methods for preparing pre-adult anadromous fish undergoing the process of smoltification for transfer to seawater. Smoltification is the stage at which a fish undergoes the acclimation or adaptation from freshwater to seawater. Smoltification also refers to a process occurring in pre-adult anadromous fish that is physiological pre-adaption to seawater while still in freshwater. The

smoltification process varies from species to species. Different species of anadromous fish can undergo smoltification at different sizes, weights, and times in the life of the fish. The present invention induces the vast majority or all of the pre-adult anadromous fish to undergo this process and be prepared for transfer to seawater.

5 The pre-adult anadromous fish are maintained in freshwater prior to adding the PVCR modulator. The term, "freshwater," means water that comes from, for example, a stream, river, ponds, public water supply, or from other non-marine sources having, for example, the following ionic composition: less than about 2 mM of magnesium, calcium and NaCl. The term "freshwater" also refers to freshwater to which at least one
10 PVCR modulator has been added, as described herein.

 The PVCR modulator is added to the freshwater in sufficient amounts to increase expression or alter the sensitivity of the PVCR. A PVCR has been isolated from various tissue of several types of anadromous fish using molecular biology techniques, as described in Example 9. DNA was isolated from muscle samples from
15 various species of anadromous fish including Atlantic Salmon, Char, Chum Salmon, Coho Salmon, King or Chinook Salmon, Pink Salmon, Sockeye Salmon and Trout. The DNA was amplified using polymerase Chain Reaction (PCR) methodology. The amplified DNA was purified and subcloned into vectors, and their sequences were determined, as described in Example 9.

20 The PVCR, which is located in various tissues (e.g., gill, skin, intestine, kidney, urinary bladder, brain or muscle) of the pre-adult anadromous fish, senses alterations in PVCR modulators including various ions (e.g., divalent cations), for example, in the surrounding water, in their serum or in the luminal contents of tubules inside the body, such as kidney, urinary bladder, or intestine. Its ability to sense these modulators
25 increases expression of the PVCR, thereby allowing the fish to better adapt to seawater. Increased expression of the PVCR can occur, for example, in one or all tissues.

 A "PVCR modulator" is defined herein to mean a compound which increases expression of the PVCR, or increases the sensitivity or responsiveness of the PVCR. Such compounds include, but are not limited to, PVCR agonists (e.g., inorganic

polycations, organic polycations and amino acids), Type II calcimimetics, and compounds that indirectly alter PVCR expression (e.g., 1,25 dihydroxyvitamin D in concentrations of about 3,000 –10,000 International Units /kg feed), cytokines such as Interleukin Beta, and Macrophage Chemotatic Peptide-1 (MCP-1)). Examples of Type

5 II calcimimetics, which increase expression and/ or sensitivity of the PVCR, are, for example, NPS-R-467 and NPS-R-568 from NPS Pharmaceutical Inc., (Salt Lake, Utah, Patent Nos. 5,962,314; 5,763,569; 5,858,684; 5,981,599; 6,001,884) which can be administered in concentrations of between about 0.1 μ M and about 100 μ M feed or water. See Nemeth, E.F. *et al.*, *PNAS* 95: 4040-4045 (1998). Examples of inorganic

10 polycations are divalent cations including calcium at a concentration between about 2.0 and about 10.0 mM and magnesium at a concentration between about 0.5 and about 10.0 mM; and trivalent cations including, but not limited to, gadolinium (Gd^{3+}) at a concentration between about 1 and about 500 μ M. Organic polycations include, but not limited to, aminoglycosides such as neomycin or gentamicin in concentrations of

15 between about 1 and about 8 gm/kg feed as well as organic polycations including polyamines (e.g., polyarginine, polylysine, polyhistidine, polyornithine, spermine, cadaverine, putricine, copolymers of poly arginine/histidine, poly lysine/arginine in concentrations of between about 10 μ M and 10 mM feed). See Brown, E.M. *et al.*, *Endocrinology* 128: 3047-3054 (1991); Quinn, S.J. *et al.*, *Am. J. Physiol.* 273: C1315-

20 1323 (1997). Additionally, PVCR agonists include amino acids such as L-Tryptophan L-Tyrosine, L-Phenylalanine, L-Alanine, L-Serine, L-Arginine, L-Histidine, L-Leucine, L-Isoleucine, and L-Cystine at concentrations of between about 1 and about 10 gm/kg feed. See Conigrave, A.D., *et al.*, *PNAS* 97: 4814-4819 (2000). The molar concentrations refer to free or ionized concentrations of the PVCR modulator in the

25 freshwater, and does not include amounts of bound PVCR modulator (e.g., PVCR modulator bound to negatively charged particles including glass, proteins, or plastic surfaces). Any combination of these modulators can be added to the water or to the feed (in addition to the NaCl, as described herein), so long as the combination increases expression and/or sensitivity of the PVCR.

The PVCR modulator can be administered to the fish in a number of ways. The invention encompasses administration of the PVCR in any way that is sufficient to increase the expression and/or alter the sensitivity of the PVCR. In one embodiment, the PVCR modulator is simply added to the freshwater in various concentrations, as described herein. A freshwater environment having at least one PVCR modulator is referred to herein as a "PVCR modulator environment." PVCR modulators that are added to the water increase expression and/or alter the sensitivity of the PVCR on the skin and gills of the fish, and can be ingested by the fish, in particular, when fish are fed feed having between about 1% and about 10% NaCl (e.g., in concentrations between about 1 and about 10 gm/100 gm feed). In addition to adding NaCl to the feed, the PVCR modulator can also be added to the feed. Amounts and types of PVCR modulators added to the feed are also described herein. Other embodiments include subjecting the fish to the PVCR modulator by "dipping" the fish in the modulator, e.g., organic polycations. The organic polycations can be formulated in such a way as to allow the polycations to adhere to the skin and gills of the fish, in sufficient amounts to increase expression of the PVCR.

In one preferred embodiment, the present invention is practiced by adding a combination of two PVCR agonists to the freshwater. In particular, calcium and magnesium are added to the freshwater to bring the concentrations of each to between about 2.0 mM and about 10.0 mM of calcium, and between about 0.5 mM and about 10.0 mM of magnesium. In addition to adding calcium and magnesium to the water, these ranges of ion concentrations can be achieved by providing a brackish water (e.g., diluted seawater) environment for the fish.

Calcium and magnesium can come from a variety of sources, that when added to the water, the calcium and/or magnesium levels increase expression of the PVCR, and/or are within the stated ranges. Sources of calcium and magnesium can be a mixture of a variety of compounds, or each can come from a substantially uniform or pure compound. Sources of calcium include, for example, $\text{Ca}(\text{CO}_3)_2$, CaCl_2 , CaSO_4 , and

Ca(OH)_2 and sources of magnesium include, for example, MgCl_2 , MgSO_4 , MgBr_2 , and MgCO_3 .

In one embodiment, the invention includes intermittent (e.g., interrupted) as well as continuous (e.g., non-interrupted) exposure to freshwater having at least one PVCR modulator, while on the NaCl diet. Intermittent exposure to the PVCR can occur so long as the PVCR expression and/or altered sensitivity remains increased. Continuous maintenance in or exposure to freshwater having at least one PVCR modulator is shown in Examples 2 and 7.

The process of the present invention pre-conditions the fish and prepares them for transfer. The pre-adult anadromous fish are maintained in a freshwater environment having a PVCR modulator long enough to increase the expression and/or alter sensitivity of the PVCR. The length of time depends on the physiological and physical maturity of the fish. Some fish will more readily adapt to the environment, and increase their expression and/or alter the sensitivity of their PVCR, while others will need more time to do so. Factors that can influence the length of time necessary to increase the expression and/or alter sensitivity of the PVCR include, but are not limited to, size of the fish, level of PVCR expression or sensitivity, if any, prior to addition of the PVCR modulator to the freshwater, the fish's ability to excrete the PVCR modulator and ions, the fish's surface to volume ratio, etc. Therefore, the length of time the fish is maintained can range from about 7 days to several months (e.g., 7, 14, 21, 30, 45, 90 and 120 days). The fish can also be maintained indefinitely so long as the fish are maintained in freshwater having the PVCR modulator and being fed a NaCl diet. For example, salmon, trout or char weighing less than 10 gms can be maintained in freshwater having a PVCR modulator, and fed a NaCl diet for at least about 180 days, prior to transfer to seawater.

The invention further includes adding feed to the freshwater. The frequency and amounts of feed that fish are fed, are taught in the art. Generally, the fish are fed 1-3 times a day, totaling about 0.25-5.0% body weight/day. The feed has enough NaCl to contribute to a significant increased level of the PVCR modulator in the serum of the

pre-adult anadromous fish. More specifically, NaCl has at least two effects. The first occurs when sufficient amounts of NaCl is present in the feed. The presence of NaCl in the feed causes the pre-adult anadromous fish to drink more water from the surrounding environment. Second, NaCl is a direct negative PVCR modulator, and works to
5 decreases PVCR sensitivity. Despite NaCl's effect in decreasing sensitivity, it surprisingly increases PVCR expression when fish are fed a NaCl diet and the surrounding freshwater environment has at least one PVCR modulator in it. The increase in the ingestion of freshwater having PVCR modulators causes an overall increase of the serum levels of PVCR modulators.

10 The present invention also relates to an aquatic food composition. The feed contains between about 1%-10% of NaCl by weight, or between about 10,000 mg of NaCl/kg of feed and about 100,000 mg of NaCl/kg of feed (e.g., 12,000 mg/kg). The feed is referred to herein as a "NaCl diet." The NaCl can be combined with other sodium salts to confer the desired effect of increasing PVCR expression, altering PVCR
15 sensitivity and/or inducing the fish to drink more. Hence, as used herein, the term NaCl, includes a substantially pure compound, and mixtures of NaCl with other sources of sodium. The feed can further include a PVCR modulator, and in particular a PVCR agonist such as an amino acid. In one embodiment, the feed has between about 1% and about 10% NaCl by weight and an amino acid such as tryptophan in an amount between
20 about 1 and about 10 gm/kg.

The feed can be made in a number of ways, so long as the proper concentration of NaCl is present. The feed can be made, for example, by reformulating the feed, or by allowing the feed to absorb a solution having the NaCl and optionally, adding a PVCR modulator. A top dressing can be added for palatability. Example 8 describes in detail
25 one way to make the feed.

Another embodiment of the present invention includes feeding pre-adult anadromous fish feed having between 1% and 10% NaCl by weight when the fish are maintained in a freshwater environment having between about 2.0 and about 10.0 mM of calcium, and between about 0.5 mM and about 10.0 mM of magnesium. When this

embodiment of the present invention is carried out, the levels of calcium, magnesium and/or sodium in the serum of the pre-adult anadromous fish increases, as compared to identically paired fish maintained in freshwater, between about 1% and 60%, between about 1% and 40%, and between about 1% and 15 %, respectively.

5 In another embodiment, the fish, while in the freshwater having the PVCR modulator, are also exposed to a photoperiod. A photoperiod refers to exposing the fish to light (e.g., sunlight, incandescent light or fluorescent light). Preferably, the photoperiod is substantially continuous, or occurs long enough to increase growth and/or induce smotification. The photoperiod can occur for at least about 12 hours
10 within a 24 hour interval, or for longer periods such as about 14, 16, 18, 20, 22 or preferably, about 24 hours.

After being exposed to the steps of the present invention, the pre-adult anadromous fish are transferred to seawater. The term, "seawater," means water that comes from the sea, or water which has been formulated to simulate the chemical and
15 mineral composition of water from the sea. The major elemental composition of the prepared seawater preferably falls substantially within the range of the major elemental composition of the natural seawater (e.g., having the following ionic composition: greater than 30mM of magnesium, greater than about 6 mM of calcium, and greater than about 300mM NaCl). Methods of preparing artificial seawater are known in the art and
20 are described in, for instance, U.S. Pat. No. 5,351,651.

When performing the methods of the present invention on pre-adult anadromous fish, the fish exhibit significant increased growth and food consumption, as compared to pre-adult anadromous fish that are not subjected to the present invention. Upon transfer to seawater, fish that are not subjected to the steps of the present invention generally
25 experience osmotic stress, reduced or no food consumption, and even death. Osmotic stress results from differences in the osmotic pressure between the surrounding environment and body compartments of the fish. This disturbs the homeostatic equilibrium of the fish and results in decreased growth, reproductive failure and reduced resistance to disease. The fish that have been preconditioned by the steps of the present

invention do not experience a significant amount of osmotic stress, and begin feeding on or soon after transfer to seawater. As a result, the fish also grow earlier. In particular, pre-adult anadromous fish that ingested a feed having between about 1% and about 10% NaCl, and between about 1 gm and about 10 gms per kg of feed of an amino acid, exhibit a substantial increase in growth. In the experiments, the fish adapted by the present invention have shown as much as about 65% increased growth during the same interval of time, as compared to identically paired fish that did not undergo the steps of the present invention and were transferred to seawater. See Table 4 of Example 2. Elimination of low feeding or poorly feeding osmotically stressed fish in a group improves the overall feed conversion ratio of the entire group. Optimal feeding and growth after seawater transfer by all members of the group of pre-conditioned fish will permit better feed utilization and improve the overall yield of production when fish reach market size.

Similarly, the present invention allows for decreasing or reducing the time between generations of pre-adult anadromous fish. These fish begin breeding earlier because the present invention increases their growth, as described herein. Since 2-3 years are required to obtain sexually mature fish, attempts to engage in selective breeding of traits requires this 2-3 year interval before a given trait can be selected for and the fish exhibit that trait breed. Improvements in growth and time to reach maturity produced by the invention reduce the time interval required to reach sexual maturity in fish by as much as about 6 months to about 12 months. Reducing the interval for breeding allows for the production of more fish, and the improved selection of fish that possess traits other than those that are better able to adapt to seawater (e.g., select for fish that have improved taste, increased filet thickness, increased $\alpha 3$ omega fatty acid content, or fish that are more readily able to increase PVCR expression).

Prior to the present invention, anadromous fish that are transferred from freshwater to seawater are generally transferred at a particular size, referred to as "critical size." The critical size varies from species to species, but generally refers to a minimum size at which a fish can be transferred to seawater. The critical size for

salmon, trout and char is between about 50 and about 100 gms, between about 70 and about 120 gms, and greater than 100 gms, respectively. Critical sizes for Coho, King, and Sockeye Salmon are between about 10 and about 15 gms, between about 20 and 40 gms and between about 1 and about 2 gms, respectively. Chum and Pink Salmon each
5 have a critical size about less than 3 gms.

Prior to the invention, a population of pre-adult anadromous fish having attained a mean critical size were transferred to seawater. Some of the fish are physiologically ready for the transfer, while others are not. This is one of the reasons for the increased mortality rate upon transfer to seawater. The methods of the present invention
10 physiologically prepares all or mostly all of the fish for transfer to seawater by increasing PVCR expression and/or sensitivity, and/or by inducing smoltification. Greater than about 80% (e.g., 90%, 95%, 100%) undergo smoltification and are ready for transfer to seawater. In fact, in one experiment, when performing the steps of the present invention on Atlantic Salmon (e.g., subjecting the fish to a PVCR modulator
15 environment and a NaCl diet), close to 100% of the Atlantic Salmon underwent smoltification. See Example 2. Hence, the methods of the present invention include methods of preparing pre-adult anadromous fish for transfer to seawater, as well as inducing smoltification in pre-adult anadromous fish.

Since the methods of the present invention increase the expression and/or
20 sensitivity of the PVCR in pre-adult anadromous fish, they survive better when transferred to seawater. The reduced osmotic stress results in reduced mortality. In one case, certain populations of pre-adult anadromous fish that did not undergo the methods of the present invention exhibit a 100% mortality rate after transfer to seawater (see Figure 9, Example 2), while other populations of pre-adult anadromous fish that did not
25 undergo the methods of the invention have survival rates of only between about 40% and 70%. See Table I, Example 2. This occurs because the fish experience osmotic shock when transferred to seawater which has a very different ionic composition than freshwater. However, when preconditioned by the methods of the present invention, the fish exhibit a survival rate that is significantly greater than the rate for unconditioned

fish (e.g., between about 80% about 100%). In fact, when performing the present invention on Atlantic Salmon, 99% of the fish survived transfer to seawater after 5 days, as compared to 33% of fish that did not undergo the steps of the present invention in one experiment. See Table I of Example 2. Hence, the present invention embodies methods of reducing the mortality rate after pre-adult anadromous fish are transferred to seawater.

Not only is the present invention useful in reducing mortality rates after transfer to seawater, the present invention is also used to increase survival rates in freshwater prior to transfer. Prior to the discovery of the present invention, a "smolt window" existed in which the hatcheries transferred the pre-adult anadromous fish to seawater, or else the fish will die if they continue to remain in freshwater after they undergo smoltification. The PVCR modulator environment and the NaCl diet of the present invention allow the fish to continue to thrive indefinitely. The fish continue to consume feed and grow. When the present invention was performed on Atlantic Salmon, 99% of the fish survived and thrived for at least 45 days in freshwater. In contrast, only 67% of the fish that did not undergo the steps of the invention survived after 45 days in freshwater in one experiment. See Example 2.

The present invention also includes methods for transferring to seawater pre-adult anadromous fish having smaller weights, as compared to the industry recognized critical size for the particular species of fish. The methods of the present invention, as described herein, increase PVCR expression in fish that are smaller than those normally transferred to seawater, or those undergoing or about to undergo smoltification. These methods include transferring a parr, the stage of a juvenile fish prior to becoming a smolt, to seawater. Parr is a life stage of pre-adult anadromous fish that occurs after maturation of alevins or yolk sac fry. Parr or fingerlings display characteristic ovoid stripes or parr marks along their flanks, and normally undergo growth and development in freshwater prior to smoltification. The term "parr" is a term that is known in the art. As yolk sac fry continue to feed, they grow into larger parr. Parr can possess a wide range of body weights depending on conditions under which they are grown. The

weights of parr vary from species to species. Body weights for parr vary significantly with a range from about 0.5 gms to about 70 gms. Carrying out the present invention in one experiment, as described herein, results in a transfer of Atlantic Salmon parr weighing as little as between about 13% and about 18.5% of the critical size weight
5 (between about 70 and about 100 gms), or about 13 gms. Adding a PVCR modulator to the feed (e.g., an amino acid such as a tryptophan), in addition to the NaCl diet, allows seawater transfer of fish having particularly low weights. See Example 2.

The present invention additionally provides methods for transferring pre-adult anadromous fish into seawater having warmer temperatures (e.g., 14°C and 19°C), as
10 compared to water temperatures into which these fish have been transferred in the past. Since the fish experience reduced or little osmotic stress when transferred to seawater using the methods of the present invention, the fish are able to withstand transfer into higher water temperatures without exhibiting an increase in mortality rates. See Example 2.

15 The methods of the present invention also decrease the incidence of disease among the smolts and the growing salmon. Because smolts treated with the methods of the present invention experience less stress upon transfer to seawater, their immune functions are stronger, and they are less susceptible to parasitic, viral, bacterial and fungal diseases. Fish not treated with the methods described herein are more
20 susceptible to such diseases, and can serve as reservoirs of disease, capable of infecting healthy fish.

Methods of Assessment of the PVCR

The present invention includes methods of detecting the level of the PVCR to determine whether fish are ready for transfer from freshwater to seawater. Methods that
25 measure PVCR levels include several suitable assays. Suitable assays encompass immunological methods, such as FACS analysis, radioimmunoassay, flow cytometry, enzyme-linked immunosorbent assays (ELISA) and chemiluminescence assays. Any method known now or developed later can be used for measuring PVCR expression.

Antibodies reactive with the PVCR or portions thereof can be used. In a preferred embodiment, the antibodies specifically bind with the PVCR or a portion thereof. The antibodies can be polyclonal or monoclonal, and the term antibody is intended to encompass polyclonal and monoclonal antibodies, and functional fragments thereof. The terms polyclonal and monoclonal refer to the degree of homogeneity of an antibody preparation, and are not intended to be limited to particular methods of production.

In several of the preferred embodiments, immunological techniques detect PVCR levels by means of an anti-PVCR antibody (i.e., one or more antibodies). The term "anti-PVCR" antibody includes monoclonal and/or polyclonal antibodies, and mixtures thereof.

Anti-PVCR antibodies can be raised against appropriate immunogens, such as isolated and/or recombinant PVCR or portion thereof (including synthetic molecules, such as synthetic peptides). In one embodiment, antibodies are raised against an isolated and/or recombinant PVCR or portion thereof (e.g., a peptide) or against a host cell which expresses recombinant PVCR. In addition, cells expressing recombinant PVCR, such as transfected cells, can be used as immunogens or in a screen for antibody which binds receptor.

Any suitable technique can prepare the immunizing antigen and produce polyclonal or monoclonal antibodies. The art contains a variety of these methods (see e.g., Kohler et al., *Nature*, 256: 495-497 (1975) and *Eur. J. Immunol.* 6: 511-519 (1976); Milstein et al., *Nature* 266: 550-552 (1977); Koprowski et al., U.S. Patent No. 4,172,124; Harlow, E. and D. Lane, 1988, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory: Cold Spring Harbor, NY); *Current Protocols In Molecular Biology*, Vol. 2 (Supplement 27, Summer '94), Ausubel, F.M. et al., Eds., (John Wiley & Sons: New York, NY), Chapter 11, (1991)). Generally, fusing a suitable immortal or myeloma cell line, such as SP2/0, with antibody producing cells can produce a hybridoma. Animals immunized with the antigen of interest provide the antibody producing cell, preferably cells from the spleen or lymph nodes. Selective culture

conditions isolate antibody producing hybridoma cells while limiting dilution techniques produce them. Researchers can use suitable assays such as ELISA to select antibody producing cells with the desired specificity.

Other suitable methods can produce or isolate antibodies of the requisite specificity. Examples of other methods include selecting recombinant antibody from a library or relying upon immunization of transgenic animals such as mice.

According to the method, an assay can determine the level of PVCR in a biological sample. In determining the amounts of PVCR, an assay includes combining the sample to be tested with an antibody having specificity for the PVCR, under conditions suitable for formation of a complex between antibody and the PVCR, and detecting or measuring (directly or indirectly) the formation of a complex. The sample can be obtained directly or indirectly, and can be prepared by a method suitable for the particular sample and assay format selected.

In particular, tissue samples, *e.g.*, gill tissue samples, can be taken from fish after they are anaesthetized with MS-222. The tissue samples are fixed by immersion in 2% paraformaldehyde in appropriate Ringers solution corresponding to the osmolality of the fish, washed in Ringers, then frozen in an embedding compound, *e.g.*, O.C.T.TM (Miles, Inc., Elkhart, Indiana, USA) using methylbutane cooled with liquid nitrogen. After cutting 8-10 μ tissue sections with a cryostat, individual sections are subjected to various staining protocols. For example, sections are: 1) blocked with goat serum or serum obtained from the same species of fish, 2) incubated with rabbit anti-CaR or anti-PVCR antiserum, and 3) washed and incubated with peroxidase-conjugated affinity-purified goat antirabbit antiserum. The locations of the bound peroxidase-conjugated goat antirabbit antiserum are then visualized by development of a rose-colored aminoethylcarbazole reaction product. Individual sections are mounted, viewed and photographed by standard light microscopy techniques. The anti-CaR antiserum used to detect fish PVCR protein is raised in rabbits using a 23-mer peptide corresponding to amino acids numbers 214-236 localized in the extracellular domain of the RaKCaR protein. The sequence of the 23-mer peptide is: ADDDYGRPGIEKFREEAEERDIC

(SEQ ID NO.: 19) A small peptide with the sequence DDYGRPGIEKFREEAEERDICI (SEQ ID NO.: 20) or ARSRNSADGRSGDDLPC (SEQ ID NO.: 21) can also be used to make antisera containing antibodies to PVCRs. Such antibodies can be monoclonal, polyclonal or chimeric.

5 Suitable labels can be detected directly, such as radioactive, fluorescent or chemiluminescent labels. They can also be indirectly detected using labels such as enzyme labels and other antigenic or specific binding partners like biotin. Examples of such labels include fluorescent labels such as fluorescein, rhodamine, chemiluminescent labels such as luciferase, radioisotope labels such as ^{32}P , ^{125}I , ^{131}I , enzyme labels
10 such as horseradish peroxidase, and alkaline phosphatase, β -galactosidase, biotin, avidin, spin labels and the like. The detection of antibodies in a complex can also be done immunologically with a second antibody which is then detected (e.g., by means of a label). Conventional methods or other suitable methods can directly or indirectly label an antibody.

15 In performing the method, the levels of the PVCR that are distinct from the control. Increased levels or the presence of PVCR expression, as compared to a control, indicate that the fish or the population of fish from which a statistically significant amount of fish were tested, are ready for transfer to freshwater. A control refers to a level of PVCR, if any, from a fish that is not subjected to the steps of the present
20 invention, e.g., not subjected to freshwater having a PVCR modulator and/or not fed a NaCl diet. For example, Figures 13 and 18 show that fish not subjected to the present invention had no detectable PVCR level, whereas fish that were subjected to the steps of the invention had PVCR levels that were easily detected.

 The PVCRs can also be assayed by Northern blot analysis of mRNA from tissue
25 samples. Northern blot analysis from various shark tissues has revealed that the highest degree of PVCRs expression is in gill tissue, followed by the kidney and the rectal gland. There appear to be at least three distinct mRNA species of about 7 kb, 4.2 kb and 2.6 kb.

The PVCRs can also be assayed by hybridization, *e.g.*, by hybridizing one of the PVCR sequences provided herein (*e.g.*, SEQ ID NO: 1,3,5,7,9,11,13,15) or an oligonucleotide derived from one of the sequences, to a DNA-containing tissue sample from a fish. Such a hybridization sequence can have a detectable label, *e.g.*, radioactive, fluorescent, etc., attached, to allow to detection of hybridization product. Methods for hybridization are well known, and such methods are provided in U.S. Pat. No. 5,837,490, by Jacobs *et al.*, the entire teachings of which are herein incorporated by reference in their entirety. The design of the oligonucleotide probe should preferably follow these parameters: (a) it should be designed to an area of the sequence which has the fewest ambiguous bases ("N's"), if any, and (b) it should be designed to have a T_m of approx. 80°C (assuming 2°C for each A or T and 4 degrees for each G or C).

Stringency conditions for hybridization refers to conditions of temperature and buffer composition which permit hybridization of a first nucleic acid sequence to a second nucleic acid sequence, wherein the conditions determine the degree of identity between those sequences which hybridize to each other. Therefore, "high stringency conditions" are those conditions wherein only nucleic acid sequences which are very similar to each other will hybridize. The sequences can be less similar to each other if they hybridize under moderate stringency conditions. Still less similarity is needed for two sequences to hybridize under low stringency conditions. By varying the hybridization conditions from a stringency level at which no hybridization occurs, to a level at which hybridization is first observed, conditions can be determined at which a given sequence will hybridize to those sequences that are most similar to it. The precise conditions determining the stringency of a particular hybridization include not only the ionic strength, temperature, and the concentration of destabilizing agents such as formamide, but also on factors such as the length of the nucleic acid sequences, their base composition, the percent of mismatched base pairs between the two sequences, and the frequency of occurrence of subsets of the sequences (*e.g.*, small stretches of repeats) within other non-identical sequences. Washing is the step in which conditions are set so as to determine a minimum level of similarity between the sequences hybridizing with

each other. Generally, from the lowest temperature at which only homologous hybridization occurs, a 1% mismatch between two sequences results in a 1°C decrease in the melting temperature (T_m) for any chosen SSC concentration. Generally, a doubling of the concentration of SSC results in an increase in the T_m of about 17°C.

- 5 Using these guidelines, the washing temperature can be determined empirically, depending on the level of mismatch sought. Hybridization and wash conditions are explained in *Current Protocols in Molecular Biology* (Ausubel, F.M. *et al.*, eds., John Wiley & Sons, Inc., 1995, with supplemental updates) on pages 2.10.1 to 2.10.16, and 6.3.1 to 6.3.6.
- 10 High stringency conditions can employ hybridization at either (1) 1x SSC (10x SSC = 3 M NaCl, 0.3 M $\text{Na}_3\text{-citrate}\cdot 2\text{H}_2\text{O}$ (88 g/liter), pH to 7.0 with 1 M HCl), 1% SDS (sodium dodecyl sulfate), 0.1 - 2 mg/ml denatured calf thymus DNA at 65°C, (2) 1x SSC, 50% formamide, 1% SDS, 0.1 - 2 mg/ml denatured calf thymus DNA at 42°C, (3) 1% bovine serum albumen (fraction V), 1 mM $\text{Na}_2\text{-EDTA}$, 0.5 M NaHPO_4 (pH 7.2)
- 15 (1 M NaHPO_4 = 134 g $\text{Na}_2\text{HPO}_4\cdot 7\text{H}_2\text{O}$, 4 ml 85% H_3PO_4 per liter), 7% SDS, 0.1 - 2 mg/ml denatured calf thymus DNA at 65°C, (4) 50% formamide, 5x SSC, 0.02 M Tris-HCl (pH 7.6), 1x Denhardt's solution (100x = 10 g Ficoll 400, 10 g polyvinylpyrrolidone, 10 g bovine serum albumin (fraction V), water to 500 ml), 10% dextran sulfate, 1% SDS, 0.1 - 2 mg/ml denatured calf thymus DNA at 42°C, (5) 5x
- 20 SSC, 5x Denhardt's solution, 1% SDS, 100 µg/ml denatured calf thymus DNA at 65°C, or (6) 5x SSC, 5x Denhardt's solution, 50% formamide, 1% SDS, 100 µg/ml denatured calf thymus DNA at 42°C, with high stringency washes of either (1) 0.3 - 0.1x SSC, 0.1% SDS at 65°C, or (2) 1 mM Na_2EDTA , 40 mM NaHPO_4 (pH 7.2), 1% SDS at 65°C. The above conditions are intended to be used for DNA-DNA hybrids of 50 base
- 25 pairs or longer. Where the hybrid is believed to be less than 18 base pairs in length, the hybridization and wash temperatures should be 5 - 10°C below that of the calculated T_m of the hybrid, where T_m in °C = (2 x the number of A and T bases) + (4 x the number of G and C bases). For hybrids believed to be about 18 to about 49 base pairs in length, the T_m in °C = (81.5°C + 16.6(log₁₀M) + 0.41(% G + C) - 0.61 (% formamide) - 500/L),

where "M" is the molarity of monovalent cations (*e.g.*, Na⁺), and "L" is the length of the hybrid in base pairs.

Moderate stringency conditions can employ hybridization at either (1) 4x SSC, (10x SSC = 3 M NaCl, 0.3 M Na₃-citrate·2H₂O (88 g/liter), pH to 7.0 with 1 M HCl),
 5 1% SDS (sodium dodecyl sulfate), 0.1 - 2 mg/ml denatured calf thymus DNA at 65°C, (2) 4x SSC, 50% formamide, 1% SDS, 0.1 - 2 mg/ml denatured calf thymus DNA at 42°C, (3) 1% bovine serum albumen (fraction V), 1 mM Na₂·EDTA, 0.5 M NaHPO₄ (pH 7.2) (1 M NaHPO₄ = 134 g Na₂HPO₄·7H₂O, 4 ml 85% H₃PO₄ per liter), 7% SDS, 0.1 - 2 mg/ml denatured calf thymus DNA at 65°C, (4) 50% formamide, 5x SSC, 0.02
 10 M Tris-HCl (pH 7.6), 1x Denhardt's solution (100x = 10 g Ficoll 400, 10 g polyvinylpyrrolidone, 10 g bovine serum albumin (fraction V), water to 500 ml), 10% dextran sulfate, 1% SDS, 0.1 - 2 mg/ml denatured calf thymus DNA at 42°C, (5) 5x SSC, 5x Denhardt's solution, 1% SDS, 100 µg/ml denatured calf thymus DNA at 65°C, or (6) 5x SSC, 5x Denhardt's solution, 50% formamide, 1% SDS, 100 µg/ml denatured
 15 calf thymus DNA at 42°C, with moderate stringency washes of 1x SSC, 0.1% SDS at 65°C. The above conditions are intended to be used for DNA-DNA hybrids of 50 base pairs or longer. Where the hybrid is believed to be less than 18 base pairs in length, the hybridization and wash temperatures should be 5 - 10°C below that of the calculated T_m of the hybrid, where T_m in °C = (2 x the number of A and T bases) + (4 x the number of
 20 G and C bases). For hybrids believed to be about 18 to about 49 base pairs in length, the T_m in °C = (81.5°C + 16.6(log₁₀M) + 0.41(% G + C) - 0.61 (% formamide) - 500/L), where "M" is the molarity of monovalent cations (*e.g.*, Na⁺), and "L" is the length of the hybrid in base pairs.

Low stringency conditions can employ hybridization at either (1) 4x SSC, (10x
 25 SSC = 3 M NaCl, 0.3 M Na₃-citrate·2H₂O (88 g/liter), pH to 7.0 with 1 M HCl), 1% SDS (sodium dodecyl sulfate), 0.1 - 2 mg/ml denatured calf thymus DNA at 50°C, (2) 6x SSC, 50% formamide, 1% SDS, 0.1 - 2 mg/ml denatured calf thymus DNA at 40°C, (3) 1% bovine serum albumen (fraction V), 1 mM Na₂·EDTA, 0.5 M NaHPO₄ (pH 7.2) (1 M NaHPO₄ = 134 g Na₂HPO₄·7H₂O, 4 ml 85% H₃PO₄ per liter), 7% SDS, 0.1 - 2

mg/ml denatured calf thymus DNA at 50°C, (4) 50% formamide, 5x SSC, 0.02 M Tris-HCl (pH 7.6), 1x Denhardt's solution (100x = 10 g Ficoll 400, 10 g polyvinylpyrrolidone, 10 g bovine serum albumin (fraction V), water to 500 ml), 10% dextran sulfate, 1% SDS, 0.1 - 2 mg/ml denatured calf thymus DNA at 40°C, (5) 5x SSC, 5x Denhardt's solution, 1% SDS, 100 µg/ml denatured calf thymus DNA at 50°C, or (6) 5x SSC, 5x Denhardt's solution, 50% formamide, 1% SDS, 100 µg/ml denatured calf thymus DNA at 40°C, with low stringency washes of either 2x SSC, 0.1% SDS at 50°C, or (2) 0.5% bovine serum albumin (fraction V), 1 mM Na₂EDTA, 40 mM NaHPO₄ (pH 7.2), 5% SDS. The above conditions are intended to be used for DNA-DNA hybrids of 50 base pairs or longer. Where the hybrid is believed to be less than 18 base pairs in length, the hybridization and wash temperatures should be 5 - 10°C below that of the calculated T_m of the hybrid, where T_m in °C = (2 x the number of A and T bases) + (4 x the number of G and C bases). For hybrids believed to be about 18 to about 49 base pairs in length, the T_m in °C = (81.5°C + 16.6(log₁₀M) + 0.41(% G + C) - 0.61 (% formamide) - 500/L), where "M" is the molarity of monovalent cations (e.g., Na⁺), and "L" is the length of the hybrid in base pairs.

Hence, the present invention includes kits for the detection of the PVCR or the quantification of the PVCR having either antibodies specific for the PVCR or a portion thereof, or a nucleic acid sequence that can hybridize to the nucleic acid of the PVCR.

Alterations in the expression or sensitivity of PVCRs could also be accomplished by introduction of a suitable transgene. Suitable transgenes would include either the PVCR gene itself or modifier genes that would directly or indirectly influence PVCR gene expression. Methods for successful introduction, selection and expression of the transgene in fish oocytes, embryos and adults are described in Chen, TT *et al.*, Transgenic Fish, *Trends in Biotechnology* 8:209-215 (1990).

The present invention is further and more specifically illustrated by the following Examples, which are not intended to be limiting in any way.

EXEMPLIFICATION

Example 1. Polyvalent cation-sensing receptors (PVCRs) serve as salinity sensors in fish.

Polyvalent cation-sensing receptors (PVCRs) serve as salinity sensors in fish.

- 5 These receptors are localized to the apical membranes of various cells within the fish's body (e.g., in the gills, intestine, kidney) that are known to be responsible for osmoregulation. A full-length cation receptor (CaR) from the dogfish shark has been expressed in human HEK cells. This receptor was shown to respond to alterations in ionic compositions of NaCl, Ca²⁺ and Mg²⁺ in extracellular fluid bathing the HEK
- 10 cells. The ionic concentrations responded to encompassed the range which includes the transition from freshwater to seawater. Expression of PVCR mRNA is also increased in fish after their transfer from freshwater to seawater, and is modulated by PVCR agonists. Partial genomic clones of PVCRs have also been isolated from other fish species, including winter and summer flounder and lumpfish, by using nucleic acid
- 15 amplification with degenerate primers.

- This method was also used to isolate partial genomic clones of PVCRs for Atlantic salmon (Fig. 1), arctic char (Fig. 2) and rainbow trout (Fig. 3). The degenerate oligonucleotide primers used were 5'-TGT CKT GGA CGG AGC CCT TYG GRA TCG C-3' (SEQ ID NO:22) AND 5'-GGC KGG RAT GAA RGA KAT CCA RAC RAT
- 20 GAA G-3' (SEQ ID NO:23), where K is T or G, Y is C or T, and R is A or G. The degenerate oligos were generated by standard methodologies (Preston, G.M., 1993, "Polymerase chain reaction with degenerate oligonucleotide primers to clone gene family members," in: Methods in Mol. Biol., vol. 58, ed. A. Harwood, Humana Press, pp. 303-312). Genomic bands from these three species were amplified, purified by
- 25 agarose gel electrophoresis, ligated into an appropriate plasmid vector (salmon and arctic char species-pT7 Blue (Novagen, Madison, WI; trout used pGem-T (Promega Biotech. Madison, WI) , and transformed into an appropriate bacterial host strain salmon and arctic char - pT7 vector with NovaBlue (Novagen, Madison, WI) and trout pGEM-T used JM-109 E. coli cell which was then grown in liquid medium. The

plasmids and inserts were purified from the host cells, and sequenced. Fig. 4 shows the deduced amino acid sequences and alignment for the PVCRs from Atlantic salmon, arctic char and rainbow trout, relative to the PVCR from the kidney of the dogfish shark (*Squalus acanthias*).

5 Example 2: Survival and Growth of Pre-Adult Anadromous Fish using the Methods of the Present Invention

An important feature of current salmon farming is the placement of smolt from freshwater hatcheries to ocean netpens. Present day methods use smolt that have attained a critical size of approximately 70-110 grams body weight. The present
10 invention can either be utilized both to improve the ocean netpen transfer of standard 70-110 grams smolt as well as permit the successful ocean netpen transfer of smolt weighing only 30 grams. For standard 70-100 gram smolt, application of the invention eliminates the phenomenon known as "smolt window" and permits fish to be maintained and transferred into ocean water at 15°C or higher. Use of the invention in 30 gram or
15 smaller smolt permits greater utilization of freshwater hatchery capacities followed by successful seawater transfer to ocean netpens. In both cases, fish that undergo the steps of the invention feed vigorously within a short interval of time after transfer to ocean netpens and thus exhibit rapid growth rates upon transfer to seawater.

Figure 5 shows in schematic form the key features of current aquaculture of
20 Atlantic salmon in ocean temperatures present in Europe and Chile. Eggs are hatched in inland freshwater hatcheries and the resulting fry grow into fingerlings and parr. Faster growing parr are able to undergo smoltification and placement in ocean netpens as S0 smolt (70 gram) during year 01. In contrast, slower growing parr are smoltified in year 02 and placed in netpens as S1 smolt (100 gram). In both S0 and S1 transfers to
25 seawater, the presence of cooler ocean and freshwater temperatures are desired to minimize the stress of osmotic shock to newly transferred smolt. This is particularly true for S1 smolt since freshwater hatcheries are often located at significant distances from ocean netpen growout sites and their water temperatures rise rapidly during early

summer. Thus, the combination of rising water temperatures and the tendency of smolt to revert or die when held for prolonged intervals in freshwater produces a need to transfer smolt into seawater during the smolt window.

Standard smolts that are newly placed in ocean netpens are not able to grow optimally during their first 40-60 day interval in seawater because of the presence of osmotic stress that delays their feeding. This interval of osmotic adaptation prevents the smolts from taking advantage of the large number of degree days present immediately after either spring or fall placement. The combination of the presence of the smolt window together with delays in achieving optimal smolt growth prolong the growout interval to obtain market size fish. This is particularly problematic for S0's since the timing of their harvest is complicated by the occurrence of grilising in maturing fish that are exposed to reductions in ambient photoperiod.

Methods

The following examples refer to APS Process I and APS Process II throughout. APS stands for "AquaBio Products Sciences®, L.L.C." APS Process I is also referred to herein as "SUPERSMOLT™ I Process" or "Process I." An "APS Process I" fish or smolt refers to a fish or smolt that has undergone the steps of APS Process I. An APS Process I smolt is also referred to as a "SUPERSMOLT™ I" or a "Process I" smolt. Likewise, APS Process II is also referred to herein as "SUPERSMOLT™ II Process" or "Process II." An "APS Process II" fish or smolt refers to a fish or smolt that has undergone the steps of APS Process II. An APS Process II smolt is also referred to as a "SUPERSMOLT™ II" or a "Process II" smolt.

APS Process I: Pre-adult anadromous fish (this includes both commercially produced S0, S1 or S2 smolts as well as smaller parr/smolt fish) are exposed to or maintained freshwater containing either 2.0-10.0 mM Calcium and 0.5-10.0 mM Magnesium ions. This water is prepared by addition of calcium carbonate and/or chloride and magnesium chloride to the freshwater. Fish are fed with feed pellets containing 7% (weight/weight) NaCl. See Example 8 for further details regarding the

feed. Fish are exposed to or maintained in this regimen of water mixture and feed for a total of 30-45 days, using standard hatchery care techniques. Water temperatures vary between 10-16°C. Fish are exposed to a constant photoperiod for the duration of APS Process I. A fluorescent light is used for the photoperiod.

- 5 APS Process II: Pre-adult anadromous fish (this includes both commercially produced S0, S1 or S2 smolts as well as smaller parr/smolt fish) are exposed to or maintained in freshwater containing 2.0-10.0 mM Calcium and 0.5-10.0 mM Magnesium ions. This water is prepared by addition of calcium carbonate and/or chloride and magnesium chloride to the freshwater. Fish are fed with feed pellets
- 10 containing 7% (weight/weight) NaCl and either 2 gm or 4 gm of L-Tryptophan per kg of feed. See Example 8 for further details regarding the feed. Fish are exposed to or maintained in this regimen of water mixture and feed for a total of 30-45 days using standard hatchery care techniques. Water temperatures vary between 10-16°C. Fish are exposed to a constant photoperiod for the duration of APS Process II. A fluorescent
- 15 light is used for the photoperiod.

Results and Discussion:

SECTION I: Demonstration of the Benefits of the APS Process I For Atlantic Salmon, Trout and Arctic Char.

Demonstration of the benefits of the APS Process I For Atlantic Salmon:

- 20 APS Process I increases the survival of small Atlantic Salmon S2 smolt after their transfer to seawater when compared to matched freshwater controls. Optimal survival is achieved by using the complete process consisting of both the magnesium and calcium water mixture as well as NaCl diet. In contrast, administration of calcium and magnesium either via the food only or without NaCl dietary supplementation does
- 25 not produce results equivalent to APS Process I.

Table 1 shows data obtained from Atlantic salmon S2 smolts less than 1 year old weighing approximately 25 gm. This single group of fish was apportioned into 4 specific groups as indicated below and each were maintained under identical laboratory conditions except for the variables tested. All fish were maintained at a water

5 temperature of 9-13°C and a continuous photoperiod for the duration of the experiment.

The control freshwater group that remained in freshwater for the initial 45 day interval experienced a 33% mortality rate under these conditions such that only 67% were able to be transferred to seawater. After transfer to seawater, this group also experienced high mortality where only one half of these smolts survived. Inclusion of
10 calcium (10 mM) and magnesium (5 mM) within the feed offered to smolt (Ca²⁺/Mg²⁺+diet) reduced survival as compared to controls both in freshwater (51% vs 67%) as well after seawater transfer (1% vs 50%). In contrast, inclusion of 10 mM Ca²⁺ and 5 mM Mg²⁺ in the freshwater (APS Process I Water Only) improved smolt survival in APS Process I water as well as after transfer of smolt to seawater. However,
15 optimal results were obtained (99% survival in both the APS Process I water mixture as well as after seawater transfer) when smolt were maintained in APS Process I water mixture and fed a diet supplemented with 7% sodium chloride.

Table 1: Comparison of the Survival of Atlantic Salmon S2 Smolts After Various Treatments

Parameter Sampled	Control Freshwater	Ca ²⁺ /Mg ²⁺ Diet	APS Water Only	APS Water + NaCl Diet
5 Starting # of fish	66	70	74	130
# of fish	44	36	67	129
10 % of fish surviving after 45 days in freshwater or APS mixture	67%	51%	91%	99%
# of fish	22	2	60	128
15 % of fish surviving 5 days after transfer to seawater	50%	1%	90%	99%
¹ Survival percentages expressed as rounded whole numbers				

20 Application of the APS Process I to the Placement of 70-100 gm smolts in seawater.

These data show that use of the APS Process I eliminates the "smolt window" and provides for immediate smolt feeding and significant improvement in smolt growth rates.

Experimental Protocol:

Smolts derived from the St. John strain of Atlantic salmon produced by the Connors Brothers Deblois Hatchery located in Cherryfield, Maine, USA were utilized for this large scale test. Smolts were produced using standard practices at this hatchery and were derived from a January 1999 egg hatching. All smolts were transferred with standard commercially available smolt trucks and transfer personnel. S1 smolt were purchased during Maine's year 2000 smolt window and smolt deliveries were taken between the dates of 29 April 2000 - 15 May 2000. Smolts were either transferred directly to Polar Circle netpens (24m diameter) located in Blue Hill Bay Maine (Controls) or delivered to the treatment facility where they were treated with APS Process I for a total of 45 days. After receiving the APS Process I treatment, the smolt were then transported to the identical Blue Hill Bay netpen site and placed in an adjacent rectangular steel cage (15mX15mX5m) for growout. Both groups of fish received an identical mixture of moist (38% moisture) and dry (10% moisture) salmonid feed (Connors Bros). Each of the netpens were fed by hand or feed blower to satiation twice per day using camera visualization of feeding. Mort dives were performed on a regular basis and each netpen received identical standard care practices established on this salmon farm. Sampling of fish for growth analyses was performed at either 42 days (APS Process I) or 120 days or greater (Control) fish. In both cases, fish were removed from the netpens and multiple analyses performed as described below.

All calculations to obtain feed conversion ratio (FCR) or specific growth rate (SGR) and growth factor (GF3) were performed using standard accepted formulae (Willoughby, S. Manual of Salmonid Farming Blackwell Scientific, Oxford UK 1999) and established measurements of degree days for the Blue Hill Bay site as provided in Table 2 below. A degree day is a measure of the number of days that a month in which a salmon can grow. It is calculated by multiplying the number of days in a month by the amount of degrees in Celsius.

Table 2: Degree days for Blue Hill Bay Salmon Aquaculture Site

Month	Degree Days
Jan	60
Feb	30
Mar	15
April	120
May	210
June	300
July	390
Aug	450
Sept	420
Oct	360
Nov	240
Dec	180

Table 3 displays data obtained after seawater transfer of Control S1 smolt. Smolt ranging from 75-125 gm were placed into 3 independent netpens and subjected to normal farm practices demonstrated characteristics typical for present day salmon aquaculture in Maine. Significant mortalities (average 3.3%) were experienced after transfer into cool (10°C) seawater and full feeding was achieved only after a significant interval (~56 days) in ocean netpens. As a result, the average SGR and GF3 values for these 3 netpens were 1.09 and 1.76 respectively for the 105-121 day interval measured.

In contrast to the immediate transfer of Control S1 smolt as described above to ocean netpens (Table III), a total of 10,600 S1 smolt possessing an average size of 63.6 grams were transported on 11 May 2000 from the Deblois freshwater hatchery to the research facility. While being maintained in standard circular tanks, these fish were

held for a total of 45 days at an average water temperature of 11°C and were subjected to APS Process I. During this interval, smolt mortality was only 64 fish (0.6%). As a matched control for the APS Process I fish, a smaller group of control fish (n=220) were held under identical conditions but did not receive the APS Process I treatment. The

5 mortalities of these control fish were minimized by the holding temperature of 10°C and were equivalent to treated smolts prior to transfer to seawater.

Table 3: Characteristics of St. John S1 smolt subjected to immediate placement in ocean netpens after transport from the freshwater hatchery without APS technology (the Control fish)

Netpen Number				
		#17	#18	#10
5	Total Fish	51,363	43,644	55,570
	Mean Date of Seawater Transfer	5/1/00	5/5/00	5/14/00
	Average Size at Transfer (grams)	(117.6) 100-125	75-100	75-100
10	Mortalities after 30 days (# and % total)	1,785; 3.5%	728; 1.7%	2503; 4.5%
	Time to achieve full feeding after transfer	68 days	48 days	50 days
15	Interval between netpen placement and analysis	121	120	105
	Average size at Analysis			
	Weight (gram)	376.8 ±74	305.80±64	298.90±37.40
20	Length (cm)	33.4±1.9	28.30±9.0	30.40±1.17
	Condition Factor (k)	1.02	1.34	1.06
	SGR	0.96	1.10	1.17
during initial 120 days				

During the 45 day interval when S1 smolts were receiving APS Process I, fish grew an average of 10 grams and thus possessed an average weight of 76.6 gm when transferred to an ocean netpen. The actual smolt transfer to seawater occurring on 26 June 2000 was notable for the unusual vigor of the smolt that would have normally been problematic since this time is well past the normal window for ocean placement of smolt. The ocean temperature at the time of APS Process I smolt netpen placement was 15.1°C. In contrast to the counterpart S1 smolts subjected to standard industry practices described above, APS Process I smolts fed vigorously within 48 hours of ocean placement and continued to increase their consumption of food during the immediate post-transfer period. The mortality of APS Process I smolts was low (6.1%) during initial 50 days after ocean netpen placement and two thirds of those mortalities were directly attributable to scale loss and other physical damage incurred during the transfer process itself.

In contrast, corresponding control fish (held under identical conditions without APS Process I treatment) did not fare well during transfer to the netpen (17% transfer mortality) and did not feed vigorously at any time during the first 20 days after ocean netpen placement. This smaller number of control fish (176) were held in a smaller (1.5mX1.5mX1.5m) netpen floating within the larger netpen containing APS Process I smolts. Their mortality post-ocean netpen placement was very high at 63% within the 51 day interval.

Both APS Process I and control smolts were fed on a daily basis in a manner identical to that experienced by the Industry Standard Fish shown on Table 3. APS Process I fish were sampled 51 days after their seawater placement and compared to the Industry Standard smolts shown on Table 2. As shown in Table 4, comparison of their characteristics reveals dramatic differences between Industry Standard smolts vs APS Process I.

Table 4: Comparison of the characteristics of St. John S1 APS Process I Smolts subjected to APS treatment and then placed in ocean netpens vs corresponding industry standard smolts.

		APS Process I Smolts	Averaged Industry Standard Data from Table 3 in this Example
5	Total Fish	10,600	150,577
	Mean Date of Seawater Transfer	6/26/00	5/7/00
	Average Size at Transfer (grams)	76.6	95.8
10	Mortalities after 30 days (# and %)	648; 6.1%	21,618;14.3%
	Time to achieve full Feeding after transfer	48 hrs	56 days
	Interval between netpen placement and analysis	51	115
15	Average size at Analysis	175.48+50	327.2
	Weight (gram)	262.22+32	30.7
	Length (cm)		
	Condition Factor (k)	0.95+0.9	1.14
	SGR	1.80	1.09

In summary, notable differences between APS Process I, Control smolt and Industry Standard smolt include:

1. The mortalities observed after ocean netpen placement were low in APS Process I (6.1%) vs Control (63%) despite the fact these fish were transferred to seawater 1.5 months after the smolt window and into a very high (15.1°C) ocean water temperature. The mortality of APS Process I was actually lower than that of Industry Standard smolt (14.3%) transferred to cooler (10°C) seawater during the smolt window. This characteristic of APS Process I provides for a greater flexibility in freshwater hatchery operations since placement of APS Process I smolts are not rigidly confined the conventional "smolt window" currently used in industry practice.

2. The APS Process I fish were in peak condition during and immediately after seawater transfer. Unlike industry standard smolt that required 56 days to reach full feeding, the APS Process I smolts fed vigorously within 48 hours. Moreover, the growth rates (SGR 1.8) demonstrated by APS Process I smolts are significantly greater than both published data for standard smolt during their initial 50 days after seawater placement (published values (Stradmeyer, L. Is feeding nonstarters a waste of time. Fish Farmer 3:12-13, 1991; Usher, ML, C Talbot and FB Eddy. Effects of transfer to seawater on growth and feeding in Atlantic salmon smolts (*Salmo salar* L.) Aquaculture 94:309-326, 1991) for SGR's range between 0.2-0.8). In fact, the growth rates of APS Process I smolts are significantly larger than Industry standard smolts placed on the same site despite the fact that industry standard smolt were both larger at the time of seawater placement as well as the fact that their growth was measured 120 days after seawater placement. These data provide evidence that the APS Process I smolts were not subjected to significant osmoregulatory stress which would prevent them from feeding immediately.

3. The rapid growth of APS Process I smolts immediately upon ocean netpen placement provides for compounding increases in the size of salmon as seawater

growout proceeds. Thus, it is anticipated that if Industry Standard Smolts weighing 112.5 gram were subjected to APS Process I treatment, placed in ocean netpens and examined at 120 days after ocean netpen placement their size would be average 782 gram instead of 377 gram as observed. This provides for more than a doubling in size of fish in the early stages of growout. Such fish would reach market size more rapidly as compared to industry standard fish.

Figure 6 provides data on the characteristics of APS Process I smolts after seawater transfer.

Application of the APS Process I to Atlantic Salmon pre-adult Fish that are Smaller than the Industry Standard "Critical Size" Smolt.

A total of 1,400 Landcatch/St John strain fingerlings possessing an average weight of 20.5 gram were purchased from Atlantic Salmon of Maine Inc., Quossic Hatchery, Quossic, Maine, USA on 1 August 2000. These fingerlings were derived from a egg hatching in January 2000 and considered rapidly growing fish. They were transported to the treatment facility using standard conventional truck transport. After their arrival, these fingerlings were first placed in typical freshwater growout conditions for 14 days. These fingerlings were then subjected to APS Process I for a total of 29 days while being exposed to a continuous photoperiod. The APS Process I were then vaccinated with the Lipogen Forte product (Aquahealth LTD.) and transported to ocean netpens by conventional truck transport and placed into seawater (15.6°C) in either a research ocean netpen possessing both a predator net as well as net openings small enough (0.25 inch) to prevent loss of these smaller APS Process I smolts. Alternatively, APS Process I smolts were placed in circular tanks within the laboratory. Forty eight hours after sea water transfer, APS Process I smolts were begun on standard moist (38% moisture) smolt feed (Connors Bros.) that had been re-pelletized due to the necessity to provide for smaller size feed for smaller APS Process I smolts, as compared to normal industry salmon. In a manner identical to that described for 70 gram smolts above, the mortality,

feed consumption, growth and overall health of these 30 gram APS Process I smolts were monitored closely.

Figure 7 displays the characteristics of a representative sample of a larger group of 1,209 APS Process I smolts immediately prior to their transfer to seawater. These parameters included an average weight of 26.6 ± 8.6 gram, length of 13.1 ± 1.54 cm and condition factor of 1.12 ± 0.06 . After seawater transfer, APS Process I smolts exhibited a low initial mortality despite the fact that their average body weight is 26-38% of industry standard 70-100 gram S0-S1 smolts. As shown in Table 5, APS Process I smolts mortality within the initial 72 hr after seawater placement was 1/140 or 0.07% for the laboratory tank. Ocean netpen mortalities after placement of APS Process I smolts were 143/1069 or 13.4%. Figure 7 shows representative Landcatch/St John strain APS Process I smolts possessing a range of body sizes that were transferred to seawater either in ocean netpens or corresponding laboratory seawater tanks. APS Process I smolts possess a wide range of sizes (46.8-5.6grams body weight) with an average body weight of 26.6 gram.

Table 5: Characteristics and survival of APS Landcatch/St. John Supersmolts I after their placement into seawater in either an APS laboratory tank or ocean netpen.

	Laboratory Tank	Ocean Netpen
Total Fish	140	1,069
Date of Seawater Transfer	9/5/00 (40); 9/12/00 (100)	9/12/00
5 Average Size at Transfer (gram)	26.6	26.6
Total mortalities after 4 days (# and % total)	1; 0.7%	143;13.4%
10 % mortality of fish weighing 25 gm and above	0; 0.0%	4; 0.4%
Time to achieve feeding	48 hrs	72 hrs

Figure 8 shows a comparison of the distributions of body characteristics for total group of Landcatch/St John APS Process I smolts vs. mortalities 72 hr after seawater ocean netpen placement . Length and body weight data obtained from the 143 mortalities occurring after seawater placement of 1,069 APS Process I smolts were plotted on data obtained from a 100 fish sampling as shown previously in Figure 7. Note that the mortalities are distributed among the smaller fish within the larger APS Process I netpen population.

Length and weight measurements for all mortalities collected from the bottom of the ocean netpen were compared to the distribution of APS Process I smolt body characteristics obtained from analysis of a representative sample prior shown in Figure 8. The data show that the mortalities occurred selectively amongst APS Process I smolts possessing small body sizes such that the mean body weight of mortalities was 54% of the mean body weight of the total transfer population (14.7/27gram or 54%).

Thus, the actual mortality rates of APS Process I smolts weighing 25-30 gram is 0.4% (4/1069) and those weighing 18-30gram is 2.9% (31/1069).

Application of APS Process I to Trout pre-adult Fish that are Smaller than the Industry Standard "Critical Size" Smolt.

- 5 Table 6 displays data on the use of the APS Process I on small (3-5 gm) rainbow trout. Juvenile trout are much less tolerant of abrupt transfers from freshwater to seawater as compared to juvenile Atlantic salmon. As a result, many commercial seawater trout producers transfer their fish to brackish water sites located in estuaries or fresh water lenses or construct "drinking water" systems to provide fresh water for trout
- 10 instead of the full strength seawater present in standard ocean netpens. After a prolonged interval of osmotic adaptation, trout are then transferred to more standard ocean netpen sites to complete their growout cycle. In general, trout are transferred to these ocean sites for growout at body weights of approximately 70-90 or 90-120 gram.

Table 6: Comparison of the Survival of Rainbow Trout (3-5 gm) in Seawater After Various Treatments.

Percent Survival of Fish ¹				
Hours Post Seawater Transfer	Control Freshwater	Constant 14 day Photoperiod	Constant 14 day Photoperiod APS Process	Constant 23 day Photoperiod + APS Process
0	100	100	100	100
24	0	25	80	99
48		0	70	81
72			40	68
96			30	58
120			30	46
Number of Fish Per Experiment	10	20	30	80
¹ Survival percentages expressed as rounded whole numbers				

A total of 140 trout from a single pool of fish less than 1 yr old were divided into groups and maintained at a water temperature of 9-13°C and pH 7.8-8.3 for the duration of the experiment described below. When control freshwater rainbow trout are transferred directly into seawater, there is 100% mortality within 24 hr (Control Freshwater). Exposure of the trout to a constant photoperiod for 14 days results in a slight improvement in survival after their transfer to seawater. In contrast, exposure of trout to APS Process I for either 14 days or 23 days results in significant reductions in

mortalities after transfer to seawater such that 30% and 46% of the fish respectively have survived after a 5 day interval in seawater. These data demonstrate that application of the APS Process I increases in the survival of pre-adult trout that are less than 7% of the size of standard "critical size" trout produced by present day industry standard

5 techniques.

Application of the APS Process I to Arctic Char pre-adult Fish that are Smaller than the Industry Standard "Critical Size" Smolt.

Although arctic char are salmonids and anadromous fish, their tolerance to seawater transfer is far less as compared to either salmon or trout. Figure 9 shows the results of
10 exposure of smaller char (3-5 gm) to the APS Process I for a total of 14 and 30 days. All fish shown in Figure 9 were exposed to a continuous photoperiod. Transfer of char to seawater directly from freshwater results in the death of all fish within 24 hr. In contrast, treatment of char with the APS Process I for 14 and 30 days produces an increase in survival such that 33% (3/9) or 73% (22/30) respectively are still alive after
15 a 3 day exposure. These data demonstrate that the enhancement of survival of arctic char that are less than 10% of the critical size as defined by industry standard methods after their exposure to the APS Process I followed by transfer to seawater.

Figure 9 shows a comparison of survival of arctic char after various treatments. A single group of arctic char (3-5 gm) were obtained from Pierce hatcheries (Buxton, ME)
20 and either maintained in freshwater or treated with the APS Process I prior to transfer to seawater.

SECTION II: The Use of the APS Process II to Permit Successful Transfer of 10-30 gram Smolt into Seawater Netpens and Tanks.

The APS Process II protocol is utilized to treat pre-adult anadromous fish for placement into seawater at an average size of 25-30 gm or less. This method differs from the APS Process I protocol by the inclusion of L-tryptophan in the diet of pre-adult anadromous fish prior to their transfer to seawater. APS Process II further improves the osmoregulatory capabilities of pre-adult anadromous fish and provides for still further reductions in the "critical size" for Atlantic salmon smolt transfers. In summary, APS Process II reduces the "critical size" for successful seawater transfer to less than one fifth the size of the present day industry standard SO smolt.

Application of APS Process II to Atlantic Salmon Fingerlings:

St John/St John strain pre-adult fingerlings derived from a January 2000 egg hatching and possessing an average weight of 0.8 grams were purchased from Atlantic Salmon of Maine Inc. Kennebec Hatchery, Kennebec Maine on 27 April 2000. These fish were transported to the treatment facility using standard conventional truck transport. After their arrival, these parr were first grown in conventional flow through freshwater growout conditions that included a water temperature of 9.6°C and a standard freshwater parr diet (Moore-Clark Feeds). On 17 July 2000, fingerlings were begun on APS Process II for a total of 49 days while being exposed to a continuous photoperiod. APS Process II smolts were then vaccinated with the Lipogen Forte product (Aquahealth LTD.) on Day #28 (14 August 2000) of APS Process II treatment. APS Process II smolts were size graded prior to initiating APS Process II as well as immediately prior to transfer to seawater. St John/St John APS Process II smolts were transported to ocean netpens by conventional truck transport and placed into seawater (15.2°C) in either a single ocean netpen identical to that described for placement of APS Process I smolts or into laboratory tanks (15.6°C) within the research facility.

Figure 10 shows representative St. John/St John strain APS Process II smolts possessing a range of body sizes were transferred to seawater either in ocean netpens or corresponding laboratory seawater tanks. Note that these APS Process II smolts possess a wide range of body weights (3.95-28 gram) that comprised an average body weight of 11.5 gram. Figure 10 shows the characteristics of St. John/St John APS Process II smolts. The average measurements of these St. John/St. John APS Process II smolts included a body weight of 11.50 ± 5.6 gram, length of 9.6 ± 1.5 cm and condition factor of 1.19 ± 0.09 . The data displayed in Table 7 shows the outcomes for two groups of APS Process II smolts derived from a single production pool of fish after their seawater transfer into either laboratory tanks or ocean netpens. Although important variables such as the water temperatures and transportation of fish to the site of seawater transfer were identical, these 2 groups of APS Process II smolts experienced differential post seawater transfer mortalities after 5 days into laboratory tanks (10% mortality) and ocean netpens (37.7% mortality).

Table 7: Characteristics and survival of APS St. John/St. John SuperSmolts II after their placement into seawater in either a laboratory tank or ocean netpen.

	<u>Laboratory Tank</u>	<u>Ocean Netpen</u>
Total Fish	100	1,316
Seawater Transfer Date	8/31/00	9/5/00
5 Water Temperature (°C)	15.6	15.6
Size at Transfer (gram)	11.5	11.5
Total Mortalities after 5 days (# and % total)	10; 10%	496; 37.7%
10 % mortalities weighing 13 grams or greater	0; 0%	1; 0.08%
Time to achieve feeding after transfer	48 hrs	48 hrs

No apparent problems were observed with the smaller (10-30gram) APS Process II smolts negotiating the conditions that exist within the confines of their ocean netpen. This included the lack of apparent problems including the ability to school freely as well as the ability to swim normally against the significant ocean currents that are continuously present in the commercial Blue Hill Bay salmon aquaculture site. While these observations are still ongoing, these data do not suggest that the placement and subsequent growth of APS Process II smolts in ocean netpens will be comprised because of lack of ability of these pre-adult anadromous fish to swim against existing ocean currents and therefore be unable to feed or develop properly.

Figure 11 compares characteristics of survivors and mortalities of APS Process II smolts after seawater transfer to either laboratory tanks (Figure 11A) or ocean netpens (Figure 11B). Figure 11A data are derived from analyses of 100 APS Process II smolts transferred to seawater tank where all fish were killed and analyzed on Day 5. In

contrast, Figure 11B displays only mortality data from ocean netpen. In both cases, only smaller APS Process II smolts experienced mortality. Note differences in Y axis scales of Figures 11A-B.

Comparison of the average body size of those APS Process II smolts that survived seawater transfer vs. those APS Process II smolts that died shows that unsuccessful APS Process II smolts possessed significantly smaller body weights as compared to average body size of whole APS Process II smolt transfer group. Thus, the average weight of mortalities in laboratory tank (5.10 ± 2.2 gram) and ocean netpen (6.46 ± 1.5 gram) are 44% and 56% respectively the value of the average body weight possessed by the entire transfer cohort (11.5 gram). In contrast, the mortalities of APS Process II smolts with body weights greater than 13 gram is 0/100 in the laboratory tank and 1/1316 or 0.076% for ocean netpens. Together, these data demonstrate that APS Process II is able to redefine the "critical size" of Atlantic salmon smolts from 70-100 gram to approximately 13 gram.

15 Application of the APS Process II to Rainbow Trout

Expansion of trout farming has been hampered by several factors. These include the fact that juvenile trout are much less tolerant of abrupt transfers from freshwater to seawater as compared to juvenile Atlantic salmon. As a result, many commercial seawater trout producers transfer their fish to brackish water sites located in estuaries or fresh water lenses or construct "drinking water" systems to provide fresh water for trout instead of the full strength seawater present in standard ocean netpens. After a prolonged interval of osmotic adaptation, trout are then transferred to more standard ocean netpen sites to complete their growout cycle. In general, trout are transferred to these ocean sites for growout at body weights of approximately 70-90 or 90-120 gram.

25 A total of 2,000 Donaldson strain trout with an average weight of 18 gram were obtained from a local commercial hatchery source (Pine Tree Trout Farm, Sanford, Maine, USA). They were derived from a December 1999 egg hatching and were

transferred from freshwater to the APS Process II at 11-12°C while being exposed to a continuous photoperiod. The total duration of APS Process II treatment was 35 days (21 June - 26 July, 2000). After being vaccinated using Lipogen Forte (Aquahealth LTD), trout were transferred directly to a research netpen containing full strength
 5 seawater at 15.6°C using standard transfer procedures as described for Atlantic salmon above. The average weight for the total group of Trout APS Process II was 22.7 gram as shown on Table 8.

Mortality counts performed identically to those described for Atlantic salmon transfers revealed a total of 513/1190 or 43.1% during the initial 5-day interval. The
 10 average body weight of these mortalities was 15.5±1.5 gram as shown on Figure 12. In a manner similar to that displayed by Atlantic salmon APS Process I and II smolts, mortalities occurred amongst the smaller trout APS Process II smolts while the larger fish exhibited little or no deaths. Thus, the average body weight for the mortality population was 15.5 gram or 68.3% of the value for total population of trout transferred
 15 to seawater. Feeding of trout was observed upon offering moist diet feed at 48 hours after placement in full strength seawater.

Table 8: Characteristics and Survival of Donaldson Rainbow Trout SuperSmolts II After Their Direct Placement into Full Strength Seawater in APS Ocean Netpen.

	Trout APS SuperSmolts II
Total Fish	1,190
20 Date of Seawater Transfer	7/25/00
Average Size at Transfer (grams)	22.7 grams
Mortalities after 5 days (# and % totla)	513; 43.1%
Average Size of Morts (grams)	15.5±1.52
Average Size of Survivors (grams)	29.35±8.3
25 Time to achieve feeding after transfer	48 hours

Figure 12 shows a distribution of body weights and lengths amongst mortalities of trout APS Process II smolts during the initial 5 days after transfer to ocean netpens. Note that the average weight of these 515 mortalities is 15.5+1.5 gram.

In summary, these data demonstrate that the benefits of the present invention are not confined to Atlantic salmon but also occur using rainbow trout. Application of the APS Process II smolts process has significantly reduced the "critical size" of rainbow trout for direct seawater transfer to approximately 30 gram. Moreover, it has eliminated the necessity for the transfer of rainbow trout into brackish water. Thus, application of the APS Process II promises to greatly expand the possible number of sites that can be utilized for full strength seawater transfer of rainbow trout.

Quantitation of Feeding and Growth of APS Process I and II smolts after Seawater Transfer:

Landcatch/St John APS Process I smolts were offered food beginning 48 hr after their seawater transfer to either laboratory tanks or ocean netpens. While these APS Process I smolts that were transferred to laboratory tanks began to feed after 48 hr, those fish transferred to ocean netpens were not observed to feed substantially until 7 days. To validate these observations, the inventors performed direct visual inspection of the gut contents from a representative sample of 49 APS Process I smolts 4 days after their seawater transfer to laboratory tanks. A total of 21/49 or 42.9% possessed food within their gut contents at that time.

The St John/St John APS Process II smolts fed vigorously when first offered food 48 hrs after their seawater transfer regardless of whether they were housed in laboratory tanks or ocean netpens. An identical direct analysis of APS Process II smolts gut contents performed as described above revealed that 61/83 or 73.5% of fish were feeding 4 days after transfer to seawater. The vigorous feeding activity of APS Process II smolts in an ocean netpen as well as laboratory tanks occurred. Taken together, these

data suggest that APS Process I and II smolts do not suffer from a prolonged (20-40 day) interval of poor feeding after seawater transfer as is notable for the much larger industry standard Atlantic salmon smolts not treated with the process.

Due to the fact that these groups of pre-adult Atlantic salmon subjected to either APS Process I or APS Process II have been transferred very recently to seawater, it is not possible to report on their ocean netpen growth rates, as shown for larger S1 smolts (Table 4). However, APS has quantified the growth rates of identical fish treated with either APS Process I or II within laboratory seawater tanks. As shown in Table 9, both Atlantic salmon treated with APS Process I or II grow rapidly during the initial interval after transfer to seawater. In contrast to industry standard smolt weighing 70-100 grams that eat poorly and thus have little or no growth during their first 20-30 days after transfer to seawater, pre-adult Atlantic salmon receiving APS Process I or II both exhibited substantial weight gains and growth despite the fact that they are only 27-38% (APS Process I) and 12-16% (APS Process II) for the critical size of industry standard smolts.

Table 9: Comparison of Growth Rates of Pre-adult Atlantic Salmon Exposed to either APS Process I or APS Process II and Placed in Laboratory Tanks During Initial Interval After Seawater Transfer

	APS Process I	APS Process II
Number of Fish	140	437
5 Weight at Placement into Seawater	26.6	11.50
Days in Seawater	22	21
Placement Weight Corrected for Mortalities	26.6*	13.15*
10 Weight after Interval in Seawater	30.3	15.2
Weight Gained in Seawater	3.75	2.05
SGR (% body weight/day)	0.60	0.68
15 FCR	1.27	2.04

* Weight gain corrected for selective mortalities amongst smaller fish (4/140 or 2.9% APS Process I; 103/437 or 23.6% APS Process II)

Example 3. Exposure of salmon smolts to Ca^{2+} and Mg^{2+} increases expression of PVCR.

20 In smolts that were exposed to 10 mM Ca^{2+} and 5.2 mM Mg^{2+} , the expression of PVCR was found to increase in a manner similar to that in smolts that are untreated, but are transferred directly to seawater.

Tissues were taken from either Atlantic salmon or rainbow trout, after anesthetizing the animal with MS-222. Samples of tissues were then obtained by
25 dissection, fixed by immersion in 3% paraformaldehyde, washing in Ringers then frozen

in an embedding compound, e.g., O.C.T.TM (Miles, Inc., Elkhart, Indiana, USA) using methylbutane cooled on dry ice. After cutting 8 micron thick tissue sections with a cryostat, individual sections were subjected to various staining protocols. Briefly, sections mounted on glass slides were: 1) blocked with goat serum or obtained from the same species of fish, 2) incubated with rabbit anti-CaR antiserum, and 3) washed and incubated with peroxidase-conjugated affinity-purified goat antirabbit antiserum. The locations of the bound peroxidase-conjugated goat antirabbit antiserum were visualized by development of a rose-colored aminoethylcarbazole reaction product. Individual sections were mounted, viewed and photographed by standard light microscopy techniques. The methods used to produce anti-PVCR antiserum are described below.

The results are shown in Figs. 13A - 13G, which are a set of seven photomicrographs showing immunocytochemistry of epithelia of the proximal intestine of Atlantic salmon smolts using anti-PVCR antiserum, and in Fig. 14, which is a Western blot of intestine of a salmon smolt exposed to Ca²⁺- and Mg²⁺-treated freshwater, then transferred to seawater. The antiserum was prepared by immunization of rabbits with a 16-mer peptide containing the protein sequence encoded by the carboxyl terminal domain of the dogfish shark PVCR ("SKCaR") (Nearing, J. et al., 1997, J. Am. Soc. Nephrol. 8:40A). Specific binding of the anti-PVCR antibody is indicated by aminoethylcarbazole (AEC) reaction product.

Figs. 13A and 13B show stained intestinal epithelia from smolts that were maintained in freshwater then transferred to seawater and held for an interval of 3 days. Abundant PVCR immunostaining is apparent in cells that line the luminal surface of the intestine. The higher magnification (1440X) shown in Fig. 13B displays PVCR protein localized to the apical (luminal-facing) membrane of intestinal epithelial cells. The pattern of PVCR staining is localized to the apical membrane of epithelial cells (small arrowheads) as well as membranes in globular round cells (arrows). Fig. 13C shows stained intestinal epithelia from a representative smolt that was exposed APS Process I and maintained in freshwater containing 10 mM Ca²⁺ and 5.2 mM Mg²⁺ for 50 days. Note that the pattern of PVCR staining resembles the pattern exhibited by epithelial

cells displayed in Figures 13A and 13B including apical membrane staining (small arrowheads) as well as larger globular round cells (arrows). Fig. 13D shows a 1900X magnification of PVCR-stained intestinal epithelia from another representative fish that was exposed to the APS Process I and maintained in freshwater containing 10 mM Ca²⁺ and 5.2 mM Mg²⁺ for 50 days and fed 1% NaCl in the diet. Again, small arrowhead and arrows denote PVCR staining of the apical membrane and globular cells respectively. In contrast to the prominent PVCR staining shown in Figures 13A-D, Figs. 13E (1440X) and 6F (1900X) show staining of intestinal epithelia from two representative smolt that were maintained in freshwater alone without supplementation of Ca²⁺ and Mg²⁺ or dietary NaCl. Both 13E and 13F display a marked lack of significant PVCR staining. Fig. 13G (1440X) shows the lack of any apparent PVCR staining upon the substitution of preimmune anti-PVCR antiserum on a section corresponding to that shown in Figure 13A where anti-PVCR antiserum identified the PVCR protein. The lack of any PVCR staining is a control to demonstrate the specificity of the anti-PVCR antiserum under these immunocytochemistry conditions.

The relative amount of PVCR protein present in intestinal epithelial cells of freshwater smolts (Figs. 13E and 13F) was negligible as shown by the faint staining of selected intestinal epithelial cells. In contrast, the PVCR protein content of the corresponding intestinal epithelial cells was significantly increased upon the transfer of these smolts to seawater (Figs. 13A and 13B). Importantly, the PVCR protein content was also significantly increased in the intestinal epithelial cells of smolts maintained in freshwater supplemented with Ca²⁺ and Mg²⁺ (Fig. 13C and 13D). The AEC staining was specific for the presence of the anti-PVCR antiserum, since substitution of the immune antiserum by the preimmune eliminated all reaction product from intestinal epithelial cell sections (Fig. 13G).

To further demonstrate the specificity of the anti-CaR antiserum to recognize salmon smolt PVCRs, Fig. 14 shows a Western blot of intestinal protein from salmon smolt maintained in 10 mM Ca²⁺, 5 mM Mg²⁺ and fed 1% NaCl in the diet. Portions of the proximal and distal intestine were homogenized and dissolved in SDS-containing

buffer, subjected to SDS-PAGE using standard techniques, transferred to nitrocellulose, and equal amounts of homogenate proteins as determined by both protein assay (Pierce Chem. Co, Rocford, IL) as well as Coomassie Blue staining were probed for presence of PVCR using standard western blotting techniques. The results are shown in the left

5 lane, labeled "CaR", and shows a broad band of about 140-160 kDa and several higher molecular weight complexes. The pattern of PVCR bands is similar to that previously reported for shark kidney (Nearing, J. et al., 1997, J. Am. Soc. Nephrol. 8:40A) and rat kidney inner medullary collecting duct (Sands, J.M. et al., 1997, J. Clin. Invest. 99:1399-1405). The lane on the right was treated with the preimmune anti-PVCR

10 antiserum used in Fig. 13G, and shows a complete lack of bands. Taken together with immunocytochemistry data shown in Figure 13, this immunoblot demonstrates that the antiserum used is specific for detecting the PVCR protein in salmon.

Example 4. Exposure of trout fingerlings to Ca^{2+} and Mg^{2+} increases expression of PVCRs.

15 Development of specific ion transport capabilities in epithelial cells of gill, kidney and intestinal tissues are important to survival of pre-adult anadromous fish if they are to survive transfer to seawater. To determine if alterations in the PVCRs accompanied the increase in trout fingerling survival in seawater, immunoblotting and immunocytochemistry was performed on samples from the fingerlings as was done for

20 the salmon smolt tissues. The results are shown in Figs. 15, 16 and 19.

Fig. 15 is an immunoblot of intestinal tissue from trout fingerlings. Anti-CaR antiserum identifies multiple bands that are specific for PVCR staining as determined by comparison of immune (lane marked CaR) vs. preimmune (lane marked pre-immune). Prominent among these bands includes a broad band of 120-160 kDa, together with

25 larger molecular weight complexes present above these bands from both intestine and gill tissue.

Figs. 16A, 16B, 16C, 16D, 16E, 16F, 16G and 16H are a set of eight photomicrographs showing immunocytochemistry of epithelia of the proximal intestine

of rainbow trout using anti-PVCR antiserum. Figs. 16A, 16C and 16E show samples from trout maintained in freshwater alone, while Figs. 16B, 16D, 16F, 16G and 16H show samples from trout maintained in freshwater supplemented with 10 mM Ca^{2+} and 5.2 mM Mg^{2+} and fed a 1% NaCl diet. Proximal intestinal segments are shown in Figs. 16A-16D, and 16G-16H, while distal intestinal segments are shown in Figs. 16E-16F. Figs. 16A-16F were treated with immune rabbit anti-CaR antiserum, washed, and developed with horseradish peroxidase-conjugated goat anti-rabbit antiserum using an aminoethylcarbazole (AEC) reaction. While Figures 16A, 16C and 16E display little or no PVCR staining, Figures 16B, 16D and 16F show significant PVCR staining that is present on the apical membrane of cells lining the intestinal lumen (small arrowheads) as well as larger globular round cells (arrows). In contrast to sections exposed to immune anti-PVCR antiserum, Figure 16H was treated with pre-immune rabbit anti-CaR antiserum and thus do not contain the colored AEC reaction product. These data indicate this method specifically detects PVCR protein bound to the anti-PVCR antiserum. Fig. 16G was stained directly with Alcian blue (Sheehan, D.C. et al., 1980, Theory and Practice of Histochemistry, Battelle Press, Columbus, Ohio, USA) to localize mucin-producing epithelial cells that are present in intestine. Note the appearance of cells staining for PVCR protein in Figures 13D (denoted by small arrows) display a similar morphological appearance to those stained with Alcian blue in Figure 13G. These data suggest that PVCR are expressed by mucin producing cells in the intestine where PVCR signaling actions modulate mucin production in the intestine.

Immunocytochemistry of intestinal tissue shows that the content of PVCR protein is different in trout maintained in freshwater alone (Figures 16A, 16C and 16E) vs. freshwater supplemented with Ca^{2+} and Mg^{2+} and trout fed a NaCl supplemented diet (Figures 16B, 16D, 16F). Normally in freshwater, CaR expression is low in either proximal (Figs. 15A and 16C) or distal (Fig. 16E) sections of intestine. However, PVCR expression is significantly increased in both proximal (Figs. 16B and 15D) and distal segments (Fig. 15F) after exposure to freshwater supplemented with 10 mM Ca^{2+} and 5.2 mM Mg^{2+} and feeding of NaCl supplemented diet.

While PVCR protein is localized to several regions of multiple cells, the presence of intense staining on the apical membranes of intestinal epithelial cells (small arrowheads) as well as occasional rounded cells (large arrowheads) are identical to data localizing PVCR protein in both the dogfish shark (Nearing, J. et al., 1997, *J. Am. Soc. Nephrol.* 8:40A), as well as rat kidney inner medullary collecting duct (IMCD) (Sands, J.M. et al., 1997, *J. Clin. Invest.* 99:1399-1405). As described above for Atlantic salmon smolts, the apical PVCR in trout intestine is induced by increases in luminal Ca^{2+} and Mg^{2+} concentrations, and thereby regulates the NaCl -mediated recovery of water from intestinal contents. This recovery is important to the survival of marine fish (Evans, D.H., 1993, "Osmotic and Ionic Regulation," in: *The Physiology of Fishes*, ed. D.H. Evans, CRC Press, Boca Raton, Florida, USA, Chapter 11, pp. 315-341), as it replaces osmotic water losses that occur via the skin and gill.

The anti-PVCR staining of rounded cells, which are interspersed throughout the larger intestinal epithelial cells (Fig. 16D) is also consistent with these cells corresponding to mucin-producing cells which are known to stain intensely with Alcian Blue (Sheehan, D.C. et al., 1980, *Theory and Practice of Histochemistry*, Battelle Press, Columbus, Ohio, USA) (Fig. 16G).

Figure 17 shows a representative immunoblot that compares the overall levels of PVCR content of protein homogenates prepared from gill tissue of trout using the same anti-PVCR antiserum as described in Figures 13-15. Prior to dissection and homogenation of gill tissue, trout were exposed to 1 of 3 different treatments including either freshwater, freshwater with 10 mM calcium and 5.2 mM magnesium with dietary NaCl supplementation or freshwater with dietary NaCl supplementation only. In the gill, the anti-PVCR antiserum also identifies a broad 120-140 kDa and a band of large molecular mass (greater than 200 kDa) that are similar to those shown in Figures 14 and 15. These data are consistent with molecular masses of CaRs of known structure and similar to those observed in immunoblotting analyses of multiple organisms, including rat (Sands, J.M. et al., 1997, *J. Clin. Invest.* 99:1399-1405), flounder, and shark (Nearing, J. et al., 1997, *J. Am. Soc. Nephrol.* 8:40A). A moderate level of PVCR

expression in gill as defined by PVCR reactive bands occurs when trout are maintained in freshwater (freshwater). The abundance of PVCR protein is increased when trout are exposed to the APS Process I (Ca, Mg + NaCl suppl. Feed) as shown in the middle lane (Ca, Mg). In contrast, when trout are maintained in freshwater and fed a NaCl diet

5 without exposure to calcium and magnesium in the freshwater, there is no change in the overall PVCR staining intensity but rather a shift of PVCR reactivity from the 120 kDa to the larger 200 kDa higher molecular weight band (lane marked salt). These data demonstrate that exposure of trout to the APS Process I (freshwater containing 10 mM calcium, 5 mM magnesium and dietary NaCl supplementation) increases PVCR

10 expression in gill tissue as compared to freshwater alone. Feeding of NaCl supplement diet while the trout are maintained in freshwater does not produce similar increased expression of the PVCR protein.

Example 5: Immunolocalization of Polyvalent Cation Receptor (PVCR) in Mucous Cells of Epidermis and in the Brain of Salmon.

15 The skin surface of salmonids is extremely important as a barrier to prevent water gain or loss depending whether the fish is located in fresh or seawater. Thus, the presence of PVCR proteins in selected cells of the fish's epidermal layer would be able to "sense" the salinity of the surrounding water as it flowed past and provide for the opportunity for continuous remodeling of the salmonid's skin based on the composition

20 of the water where it is located.

Methods: Samples of the skin from juvenile Atlantic Salmon resident in seawater for over 12 days were fixed in 3% paraformaldehyde dissolved in buffer (0.1M NaPO₄, 0.15M NaCl, 0.3M sucrose pH 7.4), manually descaled, rinsed in buffer and frozen at -80°C for cryosectioning. Ten micron sections were either utilized for

25 immunolocalization of PVCR using anti-shark PVCR antiserum or stained directly with 1% Alcian Blue dye to localize cells containing acidic glycoprotein components of mucous.

Results and Discussion: Figure 18A shows that salmon epidermis contains multiple Alcian Blue staining cells present in the various skin layers. Note that only a portion of some larger cells (that containing acidic mucins) stains with Alcian Blue (denoted by the open arrowheads). For purposes of orientation, note that scales have been removed so asterisks denote surface that was previously bathed in seawater. Figure 18B shows immunolocalization of salmon skin PVCR protein that is localized to multiple cells (indicated by arrowheads) within the epidermal layers of the skin. Note that anti-PVCR staining shows the whole cell body, which is larger than its corresponding apical portion that stains with Alcian Blue as shown in Figure 18A. The presence of bound anti-CaR antibody is indicated by the rose color reaction product. Although formal quantitation has not yet been performed on these sections, it appears that the number of PVCR cells is less than the total number of Alcian Blue positive cells. These data indicate that only a subset of Alcian Blue positive cells contain abundant PVCR protein. Figure 18C of Figure 18 shows the Control Preimmune section where the primary anti-CaR antiserum was omitted from the staining reaction. Note the absence of rose colored reaction product in the absence of primary antibody.

These data demonstrate the presence of PVCR protein in discrete epithelial cells (probably mucocytes) localized in the epidermis of juvenile Atlantic salmon. From this location, the PVCR protein could "sense" the salinity of the surrounding water and modulate mucous production via changes in the secretion of mucous or proliferation of mucous cells within the skin itself. The PVCR agonists (Ca^{2+} , Mg^{2+}) present in the surrounding water activate these epidermal PVCR proteins during the interval when smolts are being exposed to the process of the present invention. This "preconditioning" of Atlantic salmon smolts by the process of the present invention is important to increased survival of smolts after their transfer to seawater.

Localization of PVCR Protein in Brain of Atlantic Salmon:

The PVCR protein can be specifically localized to the brain stem area of Atlantic salmon using immunocytochemistry and antibody raised against a peptide sequence

found in the carboxyl terminal of the shark PVCR. These data are consistent with a role for a PVCR in the modulation of endocrine function as well as appetite control in Atlantic salmon.

Localization of the expression of calcium receptors to specific regions of the mammalian brain has been determined. While the exact functions of mammalian CaRs in many regions of the mammalian brain are still unknown, several lines of evidence indicate that CaRs can integrate alterations in systemic calcium, sodium and water metabolism with modulations in brain function that include differences in the secretion of hormones such as adrenocorticotrophin (ACTH) from the hypothalamus as well as behavioral changes such as regulation of thirst or eating. Of importance to disclosure findings detailed below, PVCRs (CaRs) roles in alteration of endocrine function, drinking and appetite in anadromous fish undergoing transfer from freshwater to seawater are important.

In mammalian brain, there is prominent CaR expression in the subfornical organ or SFO. The SFO is a key hypothalamic thirst center and is believed to play a role in modulation of drinking activity to integrate body calcium and water homeostasis. Stimulation of drinking behavior by systemic hypercalcemia via stimulation of CaRs located in the SFO is thought to minimize the dehydration produced by alterations in kidney function that blunt the tubular reabsorption of filtered water by the kidney. The equivalent SFO area of the fish brain is not presently been identified.

In the mammalian brain, there is CaR expression in the pons area of the brainstem particularly around the area postrema near the third ventricle. The area postrema is known to be collection of neurons believed to mediate appetite and has been termed the "nausea center". From this portion of the mammalian brain, neuronal pathways provide for integration of sensory input from vestibular function (sensing of balance) as well as visual input via pathways from optic nerves and their respective nuclei in the brain. This region of the brain is believed to be intimately involved in the nausea produced by hypercalcemia as well as the administration of opiates to humans. The equivalent area postrema of the fish brain is not presently identified.

A combination of physiological and anatomic data provide evidence for the role of CaRs to integrate a variety of endocrine functions with changes in the serum calcium levels in humans. Intravenous infusion of calcium sufficient to raise serum calcium concentrations causes selective increases in gonadotropic releasing hormones and thyroid releasing hormone (TRH) as well ACTH that are produced by the anterior pituitary. The anterior pituitary gland is known to be intimately connected with specific areas of the hypothalamus that express CaRs.

Increases in the serum calcium concentrations of humans cause multiple alterations in both behavior as well as endocrine function. Thus, hypercalcemia causes increased drinking, decreased food consumption and alterations in the circulating levels of specific hypothalamic hormones. As mentioned below, analogous changes in behavior and circulating hormone levels occur in preadult anadromous fish during smoltification and transfer from freshwater to seawater.

Methods:

Whole brains obtained from preadult Atlantic salmon (St. John/St John APS Process II smolts) that were subjected to the APS Process II and transferred to seawater were dissected free of their surroundings and fixed in 3% paraformaldehyde (PFA) in buffer [identical to other immunocytochemistry descriptions]. Eight micron sections were cut, attached to glass slides and processed for immunocytochemistry using either nonimmune control antiserum or anti-PVCR of dogfish shark. Specific antibody binding was detected by the rose-colored reaction product formed from the action of horseradish peroxidase conjugated goat anti-rabbit secondary antiserum and amino ethylcarbazole. Sections were viewed and photographed using standard light microscopy techniques.

Results and Discussion:

After examining serial sections from multiple preadult Atlantic salmon (average wt. 10.3 gm), there is consistent localization of PVCR protein in cells localized in 3 distinct

regions of the salmon brain. The first region of PVCR localization is distinct staining of neurons in the vagal lobe region. The second region of PVCR staining is within neurons in the commissural nucleus of Cajal. Both of these regions of salmon brain are known to represent important nuclei in the gustatory (sensing food and eating) as well as
5 general visceral activities including esophageal and intestinal motility (processing of food and intestinal contents for nutrient and water reabsorption). Expression of PVCR protein links alterations in both serum and CNS calcium concentrations to changes in eating and processing of intestinal contents important for anadromous fish adaptation to seawater.

10 A third site of PVCR localization in salmon brain is the saccus vasculosus where PVCR protein is distributed throughout multiple cell types. The saccus vasculosus is ovoid and localized on the ventral surface of the brain between the inferior lobes. This structure is highly vascularized and contains connections between the cerebral spinal fluid and the vascular space. Moreover, neurons present in the saccus vasculosus
15 possess massive nerve projections that tract to the subependymal region of the thalamus. The saccus vasculosus system modulates the function of centers of the posterior tubercle and periventricular thalamus. These areas of the brain are immediately adjacent to the pituitary gland.

The localization of PVCR protein(s) in the brain of preadult Atlantic salmon
20 provides evidence that PVCR can be involved in a variety of functions in the central nervous system of anadromous fish in a manner similar to that described above for the mammalian brain. In particular, localization of PVCR to nuclei that are part of the gustatory system in Atlantic salmon indicates that PVCR protein is expressed in neurons that modulate appetite similar to that described for the area postrema in mammals.
25 Stimulation of PVCR or alterations in its expression via changes in the serum calcium, magnesium or sodium concentrations as demonstrated for Atlantic salmon in this application would then be able to modulate appetite and food consumption. Alternatively, alterations in the cerebral spinal fluid concentration of these ions via exchange between the CSF and the vascular system can also be involved. Since

Atlantic salmon smolt produced by present day industry standard methods experience an interval of profound anorexia after their transfer to seawater, this well known suppression of appetite can be mediated through PVCR signaling mechanisms.

In a similar manner, PVCR signaling pathways can also modulate both drinking
5 behavior and pituitary hormone secretion. PVCR protein expressed in the saccus
vasculosus can provide for both the initiation of the drinking of seawater by Atlantic
salmon and can be directly analogous to increased drinking in mammals caused by
hypercalcemia. Increases in serum calcium, magnesium and sodium concentrations
produced by transfer of preadult anadromous fish from freshwater to seawater can also
10 be the stimulus for increased secretion of hypothalamic hormones such as ACTH.
ACTH stimulates the secretion of cortisol by the adrenal gland in fish. Cortisol is one
hormone that has been shown to be a modulator of ion transport activity and involved in
modulation of the parr-smolt transformation in anadromous fish. Modulation of
pituitary activity via connections between the saccus vasculosus, hypothalamus and the
15 pituitary can modulate these endocrine changes.

Example 7: Serum level in fish exposed to APS Process I or APS Process II

The data described herein demonstrates that Alterations in the Concentrations of
Calcium, Magnesium and NaCl in the Body Fluids of Anadromous Fish Occur After
Seawater Transfer and Excessively High Concentrations cause or contribute to Post
20 Seawater Transfer Deaths in Anadromous Fish. APS Process II Mimics Seawater
Transfer Without Subjecting Small Preadult Anadromous Fish to Osmotic Stress. This
"Preconditions" Fish thus Allowing Them to be Transferred to Seawater at Significantly
Smaller Sizes and Under Conditions That are Nonpermissive using Industry Standard
Practices.

25 PVCRs are present in multiple tissue locations where PVCRs are exposed to
surrounding seawater (gills, skin), luminal contents of tubules (kidney, intestine) as well
as internal body fluids (brain, endocrine tissue, muscle). When anadromous fish are
transferred from fresh to seawater there is an abrupt rise in the external water

concentrations of calcium, magnesium and NaCl. If the fish absorbs increased amounts of calcium, magnesium and NaCl via drinking or osmosis then PVCs located on the apical surfaces of intestinal and kidney epithelial cells will be exposed to increased amounts of these divalent and monovalent ions. These increases in divalent cation concentrations occur since the kidney is the primary excretory organ for divalent cations and the intestine is the major water recovery organ for anadromous fish via the processing of ingested seawater. Important for this data disclosure is the fact that if the concentrations of calcium, magnesium and NaCl increase in the blood and extracellular fluid of fish, then the PVCs that are bathed in these body fluids will become stimulated. Alterations in serum calcium and magnesium constitute an actual signaling pathway. In this regard, it is also noteworthy that there are a wide range of "normal" values for serum concentrations of calcium, sodium, magnesium and chloride in anadromous fish. While it has been recognized that steady state serum concentrations of these ions change with differing salinities, there has been no recognition that these might represent fish with differing PVC "set points" as described herein.

Current production methods for salmonids depend on the attainment of a "critical size" for preadult fish called smolt to enable them to survive the transfer from freshwater to seawater.

The production of salmonids for aquaculture is dependent on the ability for preadult fish to survive direct transfer from freshwater to seawater. For this process to occur, present day industry methods have identified a "critical size" for each species of salmonid. Below this critical size, many fish are not able to survive the dramatic alterations in water osmolality and ionic composition. Factors that contribute to the ability of "critical size" smolt include specific surface area to volume ratios as well as the maturity of ionic transport and hormonal mechanisms to cope with the new seawater ionic environment. These mechanisms involve coordinated responses from several organs including the gill, gastrointestinal tract, kidney, and skin as well as specific behavioral changes such as the initiation of drinking behavior after seawater exposure. The transfer of a fish from a freshwater to seawater environment constitutes a major

challenge to these osmoregulatory systems that are rapidly remodeled to permit its survival. The basic osmoregulatory mechanisms and responses are outlined briefly on Figure 19.

When a fish resides in freshwater, it is surrounded by an aqueous environment that possesses a significantly lower ionic and osmotic content (Table 11). Due to the osmotic gradient that exists between the body fluid of the fish and the surrounding environment, the fish is constantly gaining water that continuously threatens to dilute the more concentrated ionic content of the fish's body fluids. As a result, the freshwater fish do not drink and excrete a copious dilute urine. To prevent the loss of important body salts into the environment, the gills, gastrointestinal tract as well as kidney tubules engage in active uptake of ions from either their luminal contents or the surrounding freshwater.

Table 11: Comparison of the Ionic¹ and Osmotic² Composition of Seawater and Freshwater vs Serum (Blood) of Atlantic Salmon³

	<u>Seawater</u>	<u>Freshwater</u>	<u>Atlantic Salmon</u>
Sodium	450	0.3-5	135-185
Calcium	10	0.07-2	2.5-3.9
Magnesium	50	0.04-3	1.0-2.8
Chloride	513	0.23-10	120-138
Sulfate	26	0.05	<0.02
Osmolality	1050	1-20	330-390
¹ All values expressed as mMoles/Liter. ² Values expressed as mOsmoles/kg H ₂ O ³ Values vary whether fish is in freshwater or seawater. Range of average values provided.			

In contrast, when a fish resides in seawater the surrounding aqueous environment possess a significantly larger ionic and osmotic content as compared to the fish's own body composition (Table 11). As shown in Figure 19, marine salmonids are constantly losing body water content to the surrounding seawater. In this regard, both the integrity
5 and permeability of the fish's skin layer are important in reducing these cutaneous losses to as low as possible. To replace these ongoing water losses, the fish drinks seawater and processes it in such a way to retain water and only a portion of its constituent ions. Ingested seawater is processed by epithelial cells lining the gastrointestinal tract. In this process, the intestinal uptake of water and some NaCl by the fish is permitted while
10 Ca^{2+} and Mg^{2+} are either not absorbed or excreted by kidney tubules. Absorbed NaCl is pumped from the fish's body via gill epithelial cells.

Figure 19 compares adaptive changes present in fish in freshwater vs seawater. Specific physiological adaptations present in freshwater fish are shown schematically on the left panel. In contrast, alterations in these same physiological responses when fish
15 are in seawater are shown on the right.

It is important for the pre-adult anadromous fish to accomplish all of these adaptative changes rapidly after transfer from freshwater to seawater. Deployment and maturation of these mechanisms requires the synthesis of new proteins and remodeling of epithelial cells involved in transepithelial transport. These changes occur in a time
20 scale that will permit the smolt to survive in its new seawater environment. The smaller the fish, the larger its surface area/volume ratio. Thus, smaller fish lose their body water more rapidly and have less body water stores to buffer changes in body ionic composition. As a result, small fish rapidly lose water and they cannot replace this water via drinking seawater since their ionic removal mechanisms are not mature. As a
25 result, smaller or nonmature smolts rapidly die of electrolyte and water imbalances produced by their inability to adapt to the new osmotic and ionic environment of seawater. In contrast, larger smolts that are larger than the "critical size" possess a lower surface area to volume ratio, lose water less rapidly and have more body water to buffer ionic changes. This larger body size provides them the interval of time necessary

to deploy their more mature ionic transport mechanisms enabling them to survive.

In smolts that are either less than the critical size or possess immature physiological ion transport mechanisms, the combination of the osmotic removal of water from their bodies coupled with ingestion of ion rich seawater produces specific alterations in body fluid and electrolyte composition. These changes include: a decrease in total body water content, increases in the concentrations of calcium, magnesium and sodium chloride. Abnormally high concentrations of these monovalent and divalent cations causes a wide range of specific changes in organ and cellular functions including alterations in cellular metabolism and nerve conduction, depression of normal nervous system and muscle activity as well as cessation of normal ingestion of food and its digestion. The abnormal behavior and appearance of highly stress pre-moribund fish after seawater transfer are actually attributable to the physiological effects of elevated ions including calcium and magnesium within the body fluids of the fish.

As described herein, measurements of serum calcium, magnesium and sodium confirm these data as well as demonstrate that the present invention causes a preconditioning of physiological and ionic transport mechanisms permitting the successful seawater transfer of preadult anadromous fish that are significantly smaller than the critical size as defined by present day industry standard methods.

Methods:

Blood was obtained from fish (salmon and trout) via venipuncture into the caudal sinus and prevented from coagulation by the addition of lithium heparin. The blood was centrifuged at 4,000 rpm for 10 minutes and the resulting serum collected and stored until assay. Calcium and magnesium concentrations of 2 microliter aliquots of serum were quantified using calcium and magnesium assay kits (Kit #595, #587 Sigma Aldrich, St Louis, MO) and Na was determined by commercial testing (NorDx Laboratories, Scarborough, ME) using a Hitachi 747 analyzer.

Results and Discussion:

The APS Process II Mimics Exposure to Seawater Without the Presence of a Large Osmotic Gradient Between the Fish's Body Fluids and Surrounding Hypertonic Seawater.

5 Figure 20 shows the changes in serum calcium concentrations in juvenile trout (average body weight approximately 30 gm) subjected to seawater transfer either directly from freshwater or after exposure to various components of APS Process II. The average steady state serum calcium concentration in these trout maintained in freshwater is 2.72 ± 0.16 mM. In contrast, transfer of trout to seawater results in a
10 significant rise in serum calcium to approximately 3.80 mM within the initial 24 hr after seawater transfer. This increase in serum calcium is sustained for an interval of approximately 108 hr (4.5 days) but then declines to a slightly lower average concentration of 3.20 ± 0.42 mM by 126 hr. Thus, internal PVCs are exposed to a rise in serum calcium upon transfer of freshwater trout to seawater. The aquatic PVCs
15 would actually sense and respond to alterations in calcium this concentration range. Thus, the increase in serum calcium (a PVC agonist) likely constitutes a signal for the initiation of multiple PVC-activated processes in various organs to permitting the survival of juvenile trout in seawater.

Placement of trout in the water mixture of the present invention which contains 3
20 mM calcium and 1 mM magnesium, and feeding the trout a standard freshwater diet (Moore Clarke Feeds) results in no significant increases in serum calcium as compared to serum calcium values for trout maintained in freshwater despite the presence of a net inward gradient of calcium from external water mixture (3 mM) to internal body fluids (2.72 mM). Moreover, serum calcium concentrations of trout maintained in the water
25 mixture are not changed by alterations of the ambient photoperiod from a normal (10 hr daylight; 14 hr darkness) to continuous daylight exposure.

Figure 21 shows increases in serum calcium concentrations induced by feeding trout maintained in water mixture (3mM calcium, 1mM magnesium) a standard freshwater pelleted diet containing additional 1% sodium chloride (w/w). Feeding of

NaCl supplemented diet began immediately after determination of baseline serum calcium concentrations at time zero. Note that serum calcium concentrations became elevated after an interval of 24 hr. Data points shown represent a total of 5 or more independent determinations from a single representative experiment. Values at 24 hr and 72 hr are significantly ($p < 0.05$) increased as compared to the value at zero time.

In contrast, the feeding of trout maintained in the water mixture of the present invention with the identical standard feed except with the addition of 1% NaCl (weight/weight) produces a significant increase in serum calcium concentrations within 24 hr (Figure 21). This increase in the serum calcium concentrations of trout mimics the rise produced by transfer of trout into seawater (compare Figure 20 vs. 21). This effect of dietary NaCl to increase serum calcium levels likely occurs because the fish is obligated to excrete this excess NaCl that it has ingested. Ingestion of this excess NaCl activates the fish's drinking behavior thereby causing it to ingest water mixture containing 3 mM calcium and thereby increases its body fluid calcium content via the intestinal absorption of calcium. Ingestion of 1% NaCl alone does not alter serum calcium concentrations. Thus, the serum calcium concentration of trout maintained in freshwater (2.72 ± 0.43 $n=6$) was not altered significantly after consumption of feed containing 1% NaCl (w/w) for as long as 30 days (2.37 ± 0.25 $n=5$). These data provide a demonstration that this protocol is necessary to achieve increase the serum calcium concentrations in anadromous fish.

Transfer of Larger Atlantic Salmon Smolts Raised in Freshwater that Possess the Industry Standard "Critical Size" to Seawater Raises their Serum Calcium and Sodium Concentrations:

The data displayed in Figure 20 shows that the mean serum calcium concentration increases by approximately 40% when trout are transferred from freshwater to seawater. The magnitude of this increase is associated with significant trout mortality (approximately 30-40%) due to osmoregulatory failure in these fish that are smaller than the "critical size" for trout. In contrast, the magnitude of increases in serum calcium

concentrations is smaller (approximately 30% increase) when larger Atlantic salmon smolts that possess the critical size 60-70 gms are transferred to seawater (Figure 22A-B). During this same interval after seawater transfer, serum sodium concentrations in these same fish increase by approximately 17%. Data derived from both trout (Figure 21) and salmon (Figure 22A-B) were only collected from fish that exhibited no visible signs of stress (i.e. stressed fish exhibit body discoloration, bizarre swimming behavior or markedly decreased activity levels) during this experiment.

Figure 22 shows alterations in serum calcium (Figure 22A) and sodium (Figure 22B) after seawater transfer of S1 Atlantic salmon smolt that possess the critical size as defined by standard present day practices. Each data point represents the mean \pm S.D of 5-10 independent determinations

Figures 23A-B show post seawater transfer values for serum calcium, magnesium and sodium obtained from a cohort of 80 S1 Atlantic salmon smolt identical to that shown in Figure 22 after a total of 45 days of treatment with APS Process I (3mM Ca^{2+} , 1 mM Mg^{2+} in water) and 7% NaCl dietary supplement in food. Note that the initial serum calcium in fish exposed to APS Process I is slightly larger (2.5 vs 3.0 mM) and changes in serum concentrations of calcium and sodium are similar to those displayed in Figure 22. Moreover, calcium and magnesium do not undergo dramatic increases during the initial 120 hr interval as these fish are transferred from calcium/magnesium water mixture to seawater. In contrast, the serum sodium concentration increases approximately 12% (178.8 mM from 158.0mM) within the first 24 hr.

Taken together, these data shown in Figures 19-23 demonstrate that increases in both the serum calcium and sodium occur after transfer of preadult anadromous fish from freshwater to seawater. Moreover, the overall expression of PVCR protein increases in specific cells involved in this osmoregulatory response such as intestine. Since PVCRs are capable of responding to alterations of both calcium and sodium within these concentrations ranges, these data indicate that a new "set point" for PVCR activity is established after transfer of fish to seawater.

The data shown in Figures 21-23 demonstrate that treatment of preadult

anadromous fish with APS Process I causes increases in the serum calcium concentrations of the fish that mimic those produced by their transfer from freshwater to seawater. Exposure of the fish to the combination of calcium and magnesium in the water and NaCl in the feed causes increased calcium intake that mirrors the drinking of hypertonic seawater without the accompanying osmotic stress. Thus, the PVCs in the APS Process I fish have been "preconditioned" by their exposure to calcium and magnesium and, as a result, the fish is more readily able to adapt to seawater when it is subsequently transferred to it.

Anadromous Fish Exhibiting Visible Symptoms of Stress After Transfer to Seawater Possess Elevated Serum Values of Calcium and/or Magnesium. The Inability of Fish to Excrete These Ions is the Major Cause for Their Death After Seawater Transfer:

When pre-adult anadromous fish are transferred to seawater either directly from freshwater or after exposure to the APS Process I, some portion of the total number of fish are often unable to adapt to the dramatic differences in osmolality and ionic composition between freshwater and seawater and die of resulting electrolyte imbalances. Observations that include tracking of fish that will ultimately expire within a short time interval (24-120 hr) after seawater transfer demonstrates that they begin to exhibit visible signs of high levels of stress including alterations in their normal light silver body coloration to a darker duskier hue as well as displaying of bizarre swimming behavior or markedly decreased activity levels 24-72 hr before their death.

Comparison of serum calcium, magnesium and sodium concentrations from control nonstressed fish vs fish exhibiting signs of high levels of stress show that serum ion concentrations in stressed fish are significantly higher as compared to control (Table 12).

Table 12: Comparison of serum concentrations of juvenile Atlantic salmon and trout in seawater judged by visual inspection as either nonstressed or stressed fish.

	Serum Concentrations in mM	
	Calcium	Magnesium
Industry Standard Juvenile Trout		
Nonstressed Fish		
Stressed Fish	4.03±0.71 (n=49)	Not Done
	4.58±0.78** (n=63)	Not Done
APS Treated Atlantic Salmon		
Nonstressed Fish	3.74±0.52	2.40±0.77 (n=15)
Stressed fish	3.97±0.66	4.07±0.60** (n=16)
**P<0.01		

- 10 These signs of high stress are directly referable to abnormally elevated concentrations of calcium, magnesium and sodium ions within the body fluids of the fish. Thus, preadult anadromous fish that are unable to excrete excess divalent cations as well as process seawater to replace body water that is lost via osmosis die from the consequences of electrolyte imbalances. Anadromous fish below the critical size are not
- 15 able to rapidly adapt to the new osmotic environment of seawater and die as a result.

Exposure of Preadult Atlantic Salmon Fish Below the "Critical Size" as Defined by Present Day Industry Standard Methods to the APS Process II Prevents the Lethal Elevations of Serum Calcium, Magnesium and Sodium and Thus Allows Successful Seawater Transfer of Fish Possessing Very Small Body Weights:

- 20 Pre-adult Atlantic salmon of the St John/St John strain were begun on the APS Process II including a water mixture (3mM Ca²⁺ and 1 mM Mg²⁺) as well as feed containing a combination of 7% NaCl and 2 gm/kg (w/w) L-Tryptophan (APS Process

II) for a total of 49 days while being exposed to a continuous photoperiod. These small, but treated pre-adult Atlantic salmon (termed APS Process II smolts) were then placed into seawater into either a single ocean netpen or into laboratory tanks (15.6°C) within the research facility. Figure 24 compares the body characteristics of APS Process II smolts that adapted successfully to seawater vs APS Process II smolts from the same group that were unable to adapt to seawater and died.

As shown in Figure 24, only those Atlantic salmon preadult fish treated with the APS Process II with the smallest body weights (approximately 10%) experienced post seawater mortalities after 5 days into laboratory tanks. Comparison of the average body size of 90% surviving APS Process II smolts vs. those 10% APS Process II smolts that died shows that unsuccessful APS Process II smolts possessed smaller body weights (5.10±2.2 gm) as compared to average body size of whole APS Process II transfer group (11.5±5.65 gm). Thus, the critical size for these APS Process II smolts is approximately 13 gm. This critical body size is only 13-18.6% (13/70-100) that of the critical size defined previously by industry standard techniques. Thus, these data show that the use of the Process II has reduced the "critical size" of Atlantic salmon parr/smolt by over 80%.

Quantitation of serum calcium, magnesium and sodium concentrations in APS Process II smolts that have successfully made the transition from calcium/magnesium water mixture to seawater is shown in Figure 25. It is noteworthy that serum concentrations of calcium, magnesium or sodium did not change despite the fact that the average body size of these APS Process II smolts is less than 20% (11.5/60.5 gm) of the normal "critical size" for Atlantic Salmon smolts produced using present day industry standard methods. Figure 25 shows that neither serum calcium, magnesium or sodium concentrations increase dramatically as would be expected from data shown in Figure 20 and Table 2 as well as data published previously. Treatment of industry standard Atlantic salmon smolt/parr with APS Process II results in no dramatic increases in the concentrations of any ions measured above despite the significantly smaller body size of APS Process II smolts, as compared to large industry standard

smolts. Comparison of data shown in Figures 22A-B (Industry standard S1 smolts), Figure 23 (Industry standard S1 smolts treated with APS Process I vs Figure 25 (preadult salmon less than 20% of the industry standard critical size) also reveals that these serum concentrations for the smaller APS Process II smolts are comparable to those displayed by the larger industry standard S1 smolts. In summary, these data show that preadult Atlantic salmon treated with APS Process II do not exhibit dramatic changes in their body composition of calcium, magnesium and sodium despite their significantly smaller size. This lack of alterations in the concentrations of these ions greatly reduces stress in these fish and permits them to adapt to seawater readily.

10

Example 8: The Feed

There are two general methods to prepare feed for consumption by fish as part of APS Process I and II. These two processes involve either reformulation of feed or addition of a concentration solution for absorption by the feed followed by a top dressing for palatability. This disclosure describes the methodology to prepare feed using each of these 2 methods.

15

Methods:

Feed Manufacture for salmon experiments

To reformulate feed, the ingredients are as follows: Base Diet was made using the following ingredients and procedure: 30% Squid (liquefied in blender), 70% Corey Aquafeeds flounder diet (powderized in blender). Ingredients were blended into a semi moist "dough" ball. Other ingredients including NaCl or PVCR active compounds were blended into the base diet by weight according to what the experiment called for.

Moore Clark standard freshwater salmonid diet (sizes 1.2, 1.5, 2.0, 2.5, and 3.5 mm) can also be used. A top dressing was applied to the pellets such that top dressing is composed of 4% of the weight of the Base Diet. Top dressing is composed of 50% krill hydrolysate (Specialty Marine Products Ltd.) and 50% Menhaden fish oil. The top dressing is added for palatability and sealing of added ingredients.

25

Other ingredients can include NaCl, MgCl₂, CaCl₂ or L-Tryptophan that are added by weight to the base diet by weight.

Preparation of Feed Containing 7% (weight/weight) NaCl:

For the APS Process I: Solid sodium chloride or NaCl apportioned at a ratio of 7% of the weight of the Moore Clark standard freshwater salmonid diet weight was added to a volume of tap water approximately 3-4 times the weight of NaCl. The mixture was heated to 60-70o C with mixing via use of a magnetic stirring bar to dissolve salt. The NaCl solution was then poured into a hand held sprayer and applied to the Moore Clark standard freshwater salmonid diet that is tumbling inside of a 1.5 cubic meter motorized cement mixer. After absorption of the NaCl rich solution, the wetted Moore Clark standard freshwater salmonid diet is spread out thinly on window screening and placed in an enclosed rack system equipped with a fan and 1500 watt heater to expedite drying process. After drying for approximately 6 hr, the dried NaCl-rich pellets are returned to the cement mixer and a top dressing is applied. The feed is stored at room temperature until use.

Preparation of Feed Containing 7% (weight/weight) NaCl + PVCR Agonist (Tryptophan) For the APS Process II: Solid sodium chloride or NaCl apportioned at a ratio of 7% of the weight of the Moore Clark standard freshwater salmonid diet weight was added to a volume of tap water approximately 3-4 times the weight of NaCl. The mixture was heated to 60-70o C with mixing via use of a magnetic stirring bar to dissolve salt. USP Grade L-Tryptophan was added to the water at either 2 grams or 4 grams for every kg of Moore Clark standard freshwater salmonid diet depending on formulation need. Dilute hydrochloric acid was added to the water with mixing until the tryptophan was dissolved and the pH of solution was approximately 4.0. The NaCl + Tryptophan solution was then poured into a hand held sprayer and was then applied to the Moore Clark standard freshwater salmonid diet tumbling inside a cement mixer. After absorption of the NaCl+Tryptophan solution, the wetted Moore Clark standard

freshwater salmonid diet is then spread out thinly on window screening and placed in an enclosed rack system equipped with a fan and 1500-watt heater to expedite drying process. After drying for approximately 6 hr, the dried NaCl/Tryptophan-rich pellets are then returned to the cement mixer and a top dressing is applied. The feed is stored at
5 room temperature until use.

Example 9: DNA and Putative Protein Sequences from Partial Genomic Clones of Polyvalent Cation Receptor Protein Amplified by PCR from the DNA of 8 Species of Anadromous Fish.

These data provide the partial genomic sequences derived from the PVCR gene in 8
10 species of anadromous fish. Each of these nucleotide sequences is unique and thus could be used as a unique probe to isolate the full-length cDNA from each species. Moreover, this DNA fragment could form the basis for a specific assay kit(s) for detection of PVCR expression in various tissues of these fish.

The PVCR has been isolated in several species of salmon, char and trout. Sequences of mammalian CaRs together with the nucleotide sequence of SKCaR (Figures 28A-B) were used to design degenerate oligonucleotide primers to highly conserved regions in the extracellular domain of polyvalent cation receptor proteins using standard methodologies (See GM Preston, Polymerase chain reaction with degenerate oligonucleotide primers to clone gene family members, *Methods in Mol. Biol.* Vol. 58 Edited by A. Harwood, Humana Press, pages 303-312, 1993). Using these primers, cDNA or genomic DNA from various fish species representing important commercial products are amplified using standard PCR methodology. Amplified bands are then purified by agarose gel electrophoresis and ligated into appropriate plasmid vector that is transformed into a bacterial strain. After growth in liquid media, vectors and inserts are purified using standard techniques, analyzed by restriction enzyme analysis and sequenced where appropriate. Using this methodology, nucleotide sequences were amplified.

To generate the data displayed in Figures 26 and 27, DNA was isolated from muscle samples of each of the species indicated using standard published techniques. DNA was then amplified using polymerase chain reaction (PCR) methodology including 2 degenerate PCR primers (DSK-F3 and DSK-R4; SEQ ID NO:22 and 23). Amplified DNAs were then purified by agarose gel electrophoresis, subcloned into plasmid vectors, amplified, purified and sequenced using standard methods.

Figure 26 shows an aligned genomic DNA sequences of 594 nucleotides for 8 anadromous fish species, each of which codes for an identical region of the PVCR protein. Note that each nucleotide sequence derived from each specific species is unique. However, alterations in the DNA sequences of these genes often occur at common specific nucleotides within each sequence of 594 nucleotides.

Figure 27 shows aligned corresponding predicted protein sequences derived from genomic nucleotide sequences displayed in Figure 26. Note that only 3 alterations in the amino acid sequence of this portion of the PVCR occur as a consequence of alterations in the nucleotide sequence as shown in Figure 26. All of these changes (Ala to Val; Arg

to Lys; and Cys to Tyr) are known as "conservative" substitutions of amino acids in that they preserve some combination of the relative size, charge and hydrophobicity of the peptide sequence.

All cited references, patents, and patent applications are incorporated herein by
5 reference in their entirety. Also, companion Patent Application No. 09/687,373, entitled
"Growing Marine Fish in Fresh Water," filed on October 12, 2000; Patent Application
No. 09/687,476, entitled "Methods for Raising Pre-adult Anadromous Fish," filed on
October 12, 2000; Patent Application No. 09/687,372, entitled "Methods for Raising
Pre-adult Anadromous Fish," filed on October 12, 2000; Provisional Patent Application
10 No. 60/240,392, entitled "Polyvalent Cation Sensing Receptor Proteins in Aquatic
Species," filed on October 12, 2000; Provisional Patent Application No. 60/240,003,
entitled "Polyvalent Cation Sensing Receptor Proteins in Aquatic Species," filed on
October 12, 2000, are all hereby incorporated by reference in their entirety.
Additionally, Application No. 09/162,021, filed on September 28, 1998, International
15 PCT application No. PCT/US97/05031, filed on March 27, 1997, and Application No.
08/622,738 filed March 27, 1996, all entitled, "Polycation Sensing Receptor in Aquatic
Species and Methods of Use Thereof" are all hereby incorporated by reference in their
entirety.

While this invention has been particularly shown and described with references to
20 preferred embodiments thereof, it will be understood by those skilled in the art that
various changes can be made therein without departing from the scope of the invention
encompassed by the appended claims.